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(54) Title: MAMMALIAN WNT POLYPEPTIDE-5

(57) Abstract: Mammalian Wnt Polypeptide-5 (Zwnt5), polynucleotides that encode the polypeptides, and antibodies that specifically bind to the polypeptides. The polypeptides, polynucleotides and antibodies can be used to detect breast cancer.

MAMMALIAN WNT POLYPEPTIDE–5

BACKGROUND OF THE INVENTION

The *Wnt* genes constitute a large family of evolutionarily conserved genes that have been isolated from numerous multicellular animal species from *Caenorhabditis elegans* to human. The genes encode cysteine-rich secreted glycoproteins that act as extracellular signaling factors. *Wnt* genes have been shown to function in a wide variety of biological processes, including cell fate determination and patterning in early embryos, and in growth and/or differentiation of the brain, kidneys, limbs, somites, and mammary glands. Two of the first known *Wnt* genes, *Wnt1* and *Wnt3*, were discovered as oncogenes activated by the mouse mammary tumor virus in virus-induced carcinomas. Recent studies showing that WNT family members are overexpressed in a proportion of breast and colon cancers now indicate that WNT genes may also contribute to cancer in humans. In view of the known functional redundancy among *Wnt* genes in experimental assays, there is a particularly important need to identify remaining members of the WNT gene family, as these may represent candidate oncogenes in human neoplasia. There is also a need to discover new full-length cDNAs that can be used as teaching aids in molecular biology classes, in particular, lab classes. As each new cDNA, and polypeptide are unique, the teaching and learning value of each cDNA and polypeptide presents unique specific, substantial and unique learning experiences.

DESCRIPTION OF THE INVENTION

The present invention fills this need by providing for a new Wnt polypeptide termed "human Wnt polypeptide-5", hereinafter referred to as "Zwnt5".The present invention also provides *Zwnt5* polynucleotides that encode Zwnt5 polypeptides and Zwnt5 fusion proteins.

In particular, the present invention provides isolated polypeptides having an amino acid sequence that is at least 90% identical to an amino acid sequence selected from the group consisting of (a) amino acid residues 28-361 of SEQ ID NO: 2, (b) amino acid sequence of SEQ ID NO: 3, or (c) the amino acid sequence of SEQ ID NO: 2, wherein the isolated polypeptide either specifically binds with an antibody that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NO:2 or SEQ ID NO:3. Examples of such polypeptides include polypeptides comprising

the amino acid sequence of either SEQ ID NO: 2 or SEQ ID NO: 3. The present invention further provides pharmaceutical compositions that comprise such polypeptides, and a pharmaceutically acceptable carrier.

Additional variant human Zwnt5 polypeptides include those in which the amino acid sequence of the variant polypeptide shares an identity with the amino acid sequence of SEQ ID NO: 2 selected from the group consisting of at least 90% identity, at least 95% identity, or greater than 95% identity, and wherein any difference between the amino acid sequence of the variant polypeptide and the amino acid sequence of SEQ ID NO: 2 is due to one or more conservative amino acid substitutions.

The present invention further provides isolated nucleic acid molecules that encode SEQ ID NO: 3, in particular a nucleotide sequence comprising the nucleotide sequence of nucleotides 79 to 1083 of either SEQ ID NO: 1.

The present invention also provides vectors and expression vectors comprising such nucleic acid molecules, recombinant host cells comprising such vectors and expression vectors, and recombinant viruses comprising such expression vectors. These expression vectors and recombinant host cells can be used to prepare Zwnt5 polypeptides. In addition, the present invention provides pharmaceutical compositions, comprising a pharmaceutically acceptable carrier and at least one of such an expression vector or recombinant virus. Preferably, such pharmaceutical compositions comprise a human *Zwnt5* gene, or a variant thereof.

The present invention further contemplates antibodies and antibody fragments that specifically bind with Zwnt5 polypeptides. Such antibodies include polyclonal antibodies, murine monoclonal antibodies, humanized antibodies derived from murine monoclonal antibodies, and human monoclonal antibodies. Examples of antibody fragments include $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv, and minimal recognition units.

The present invention also provides methods for detecting the presence of *Zwnt5* RNA in a biological sample, comprising the steps of :

(a) contacting an *Zwnt5* nucleic acid probe under hybridizing conditions with either (i) test RNA molecules isolated from the biological sample, or (ii) nucleic acid molecules synthesized from the isolated RNA molecules, wherein the probe has a nucleotide sequence comprising a portion of the nucleotide sequence selected from the group consisting of SEQ ID NO:1, the complement of SEQ ID NO:1, and

(b) detecting the formation of hybrids of the nucleic acid probe and either the test RNA molecules or the synthesized nucleic acid molecules,

wherein the presence of the hybrids indicates the presence of *Zwnt5* RNA in the biological sample.

In addition, the presence of Zwnt5 polypeptide in a biological sample can be detected by methods that comprise the steps of (a) contacting the biological sample with an antibody, or an antibody fragment, that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NO: 2 or SEQ ID NO: 3, wherein the contacting is performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and (b) detecting any of the bound antibody or bound antibody fragment.

The present invention also provides kits for detecting *Zwnt5* nucleic acid molecules or Zwnt5polypeptides. For example, a kit for detection of *Zwnt5* nucleic acid molecules may comprise a container that comprises a nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of nucleotides 79 to 1083 of SEQ ID NO: 1, (b) a nucleic acid molecule comprising the complement of the nucleotide sequence of SEQ ID NO: 1, (c) a nucleic acid molecule that is a fragment of (a) consisting of at least eight nucleotides, (d) a nucleic acid molecule that is a fragment of (b) consisting of at least eight nucleotides, (e) a nucleic acid molecule comprising the nucleotide sequence of nucleotides 79 to 1083 of SEQ ID NO: 1, (f) a nucleic acid molecule comprising the complement of the nucleotide sequence of SEQ ID NO: 1, (g) a nucleic acid molecule that is a fragment of (e) consisting of at least eight nucleotides, and (h) a nucleic acid molecule that is a fragment of (f) consisting of at least eight nucleotides. Such kits may further comprise a second container that comprises one or more reagents capable of indicating the presence of the nucleic acid molecule. A kit for detection of Zwnt5 polypeptide may comprise a container that comprises an antibody, or an antibody fragment, that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NO: 2 or SEQ ID NO: 3.

The present invention also contemplates isolated nucleic acid molecules comprising a nucleotide sequence that encodes an Zwnt5 secretion signal sequence and a nucleotide sequence that encodes a biologically active polypeptide, wherein the Zwnt5ε secretion signal sequence comprises an amino acid sequence of residues 1 to 27 of SEQ ID NO: 2. Illustrative biologically active polypeptides include Factor VIIa, proinsulin, insulin, follicle-stimulating hormone, tissue type plasminogen activator, tumor necrosis factor, interleukin, colony stimulating factor, interferon, erythropoietin, and thrombopoietin. Moreover, the present invention provides fusion proteins comprising an Zwnt5 secretion signal sequence and a polypeptide, wherein the Zwnt5 secretion signal sequence comprises an amino acid sequence of residues 1 to 27 of SEQ ID NO: 2.

The present invention also contemplates anti-idiotype antibodies, or anti-idiotype antibody fragments, that specifically bind with an anti-Zwnt5 antibody or antibody fragment.

The present invention further includes methods for detecting an alteration in chromosome 17. In particular, human *Zwnt5*nucleotide sequences can be used to examine chromosome 17q, for example, in the 17q21 region, which is 7.8 cR distal to the breast cancer susceptibility gene BRCA1. Illustrative chromosomal aberrations at the *Zwnt5* gene locus include aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. These aberrations can occur within flanking sequences, including upstream promoter and regulatory regions, and can be manifested as physical alterations within a coding sequence or changes in gene expression level. Such methods are effected by examining the *Zwnt5* gene and its gene products. In general, suitable assay methods include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism analysis, short tandem repeat analysis employing polymerase chain reaction techniques, ligation chain reaction, ribonuclease protection assays, use of single-nucleotide polymorphisms, protein truncation assays, and other genetic linkage techniques known in the art.

In particular, the present invention provides methods for diagnosing an alteration in the *Zwnt5*gene of an individual, comprising: (a) amplifying nucleic acid molecules that encode *Zwnt5* from RNA isolated from a biological sample of the individual, and (b) detecting a mutation in the amplified nucleic acid molecules, wherein the presence of a mutation indicates an alteration in the *Zwnt5* gene. Similarly, methods of detecting a chromosome 17q21 abnormality in a subject comprise: (a) amplifying nucleic acid molecules that encode Zwnt5 from RNA isolated from a biological sample of the subject, and (b) detecting a mutation in the amplified nucleic acid molecules, wherein the presence of a mutation indicates a chromosome 17q21 abnormality. In variations of these methods, the detecting step is performed by comparing the nucleotide sequence of the amplified nucleic acid molecules to the nucleotide sequence of SEQ ID NO:1. Alternatively, the detecting step can be performed by fractionating the amplified nucleic acid molecules and control nucleic acid molecules that encode the amino acid sequence of SEQ ID NOs: 2 or 3, and comparing the lengths of the fractionated amplified and control nucleic acid molecules. Exemplary methods for amplification include polymerase chain reaction or reverse transcriptase-polymerase chain reaction.

The present invention also includes methods for detecting a chromosome 17q21 abnormality in a subject comprising: (a) amplifying nucleic acid molecules that encode *Zwnt5* from RNA isolated from a biological sample of the subject, (b) transcribing the amplified nucleic acid molecules to express *Zwnt5* mRNA, (c)

translating *Zwnt5* mRNA to produce Zwnt5ε polypeptides, and (d) detecting a mutation in the Zwnt5 polypeptides, wherein the presence of a mutation indicates a chromosome 17q21 abnormality. In variations of these methods, the detection step can be performed by fractionating, under denaturing conditions, the Zwnt5 polypeptides and control polypeptides that encode the amino acid sequence of SEQ ID NO: 2 or 3, and comparing the sizes of the fractionated amplified and control polypeptides. Similar methods can be used to detect a mutation of a *Zwnt5* gene in an individual.

1. Overview

The encoded polypeptide has the amino acid sequence of ID NO: 2 or SEQ ID NO: 3.

2. Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

As used herein, "nucleic acid" or "nucleic acid molecule" refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise

naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

The term "complement of a nucleic acid molecule" refers to a nucleic acid molecule having a complementary nucleotide sequence and reverse orientation as compared to a reference nucleotide sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a nucleic acid molecule that has a contiguous stretch of identical or complementary sequence to another nucleic acid molecule. Contiguous sequences are said to "overlap" a given stretch of a nucleic acid molecule either in their entirety or along a partial stretch of the nucleic acid molecule.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons as compared to a reference nucleic acid molecule that encodes a polypeptide. Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (*i.e.*, GAU and GAC triplets each encode Asp).

The term "structural gene" refers to a nucleic acid molecule that is transcribed into messenger RNA (mRNA), which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a growth factor that has been separated from the genomic DNA of a cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism. A nucleic acid molecule that has been isolated from a particular species is smaller than the complete DNA molecule of a chromosome from that species.

A "nucleic acid molecule construct" is a nucleic acid molecule, either single- or double-stranded, that has been modified through human intervention to contain segments of nucleic acid combined and juxtaposed in an arrangement not existing in nature.

"Linear DNA" denotes non-circular DNA molecules having free 5' and 3' ends. Linear DNA can be prepared from closed circular DNA molecules, such as plasmids, by enzymatic digestion or physical disruption.

"Complementary DNA (cDNA)" is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA

strand. The term "cDNA" also refers to a clone of a cDNA molecule synthesized from an RNA template.

A "promoter" is a nucleotide sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee *et al.*, *Mol. Endocrinol.* 7:551 (1993)), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, *Seminars in Cancer Biol.* 1:47 (1990)), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly *et al.*, *J. Biol. Chem.* 267:19938 (1992)), AP2 (Ye *et al.*, *J. Biol. Chem.* 269:25728 (1994)), SP1, cAMP response element binding protein (CREB; Loeken, *Gene Expr.* 3:253 (1993)) and octamer factors (see, in general, Watson *et al.*, eds., *Molecular Biology of the Gene*, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre and Rousseau, *Biochem. J.* 303:1 (1994)). If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known.

A "core promoter" contains essential nucleotide sequences for promoter function, including the TATA box and start of transcription. By this definition, a core promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity.

A "regulatory element" is a nucleotide sequence that modulates the activity of a core promoter. For example, a regulatory element may contain a nucleotide sequence that binds with cellular factors enabling transcription exclusively or preferentially in particular cells, tissues, or organelles. These types of regulatory elements are normally associated with genes that are expressed in a "cell-specific," "tissue-specific," or "organelle-specific" manner. For example, the Zwnt5 regulatory element preferentially induces gene expression in placental, tracheal, and uterine tissues, as opposed to lung, brain, liver, kidney, spleen, thymus, prostate, testis, ovary, small intestine, and pancreas tissues.

An "enhancer" is a type of regulatory element that can increase the efficiency of transcription, regardless of the distance or orientation of the enhancer relative to the start site of transcription.

"Heterologous DNA" refers to a DNA molecule, or a population of DNA molecules, that does not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (*i.e.*, endogenous DNA) so long as that host DNA is combined with non-host DNA (*i.e.*, exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a transcription promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous gene operably linked with an exogenous promoter. As another illustration, a DNA molecule comprising a gene derived from a wild-type cell is considered to be heterologous DNA if that DNA molecule is introduced into a mutant cell that lacks the wild-type gene.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides."

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

An "integrated genetic element" is a segment of DNA that has been incorporated into a chromosome of a host cell after that element is introduced into the cell through human manipulation. Within the present invention, integrated genetic elements are most commonly derived from linearized plasmids that are introduced into the cells by electroporation or other techniques. Integrated genetic elements are passed from the original host cell to its progeny.

A "cloning vector" is a nucleic acid molecule, such as a plasmid, cosmid, or bacteriophage that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites that allow insertion of a nucleic acid molecule in a determinable fashion without loss of an essential biological function of the vector, as well as nucleotide sequences encoding a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

An "expression vector" is a nucleic acid molecule encoding a gene that is expressed in a host cell. Typically, an expression vector comprises a transcription promoter, a gene, and a transcription terminator. Gene expression is usually placed under the control of a promoter, and such a gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

A "recombinant host" is a cell that contains a heterologous nucleic acid molecule, such as a cloning vector or expression vector. In the present context, an example of a recombinant host is a cell that produces Zwnt5from an expression vector. In contrast, Zwnt5 can be produced by a cell that is a "natural source" of Zwnt5, and that lacks an expression vector.

"Integrative transformants" are recombinant host cells, in which heterologous DNA has become integrated into the genomic DNA of the cells.

A "fusion protein" is a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes. For example, a fusion protein can comprise at least part of a Zwnt5 polypeptide fused with a polypeptide that binds an affinity matrix. Such a fusion protein provides a means to isolate large quantities of Zwnt5 using affinity chromatography.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule termed a "ligand." This interaction mediates the effect of the ligand on the cell. Receptors can be membrane bound, cytosolic or nuclear; monomeric (*e.g.*, thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (*e.g.*, PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor). Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. In certain membrane-bound receptors, the extracellular ligand-binding domain and the intracellular effector domain are located in separate polypeptides that comprise the complete functional receptor.

In general, the binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell, which in turn leads to an alteration in the metabolism of the cell. Metabolic events that are often linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids.

The term "secretory signal sequence" denotes a DNA sequence that encodes a peptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, *i.e.*, at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel. However, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The terms "amino-terminal or N-terminal" and "carboxyl-terminal or C-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "expression" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a polypeptide encoded by a splice variant of an mRNA transcribed from a gene.

As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors, lymphotoxins, co-stimulatory molecules, hematopoietic factors, and synthetic analogs of these molecules.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of less than 10^9 M^{-1}.

An "anti-idiotype antibody" is an antibody that binds with the variable region domain of an immunoglobulin. In the present context, an anti-idiotype antibody binds with the variable region of an anti-Zwnt5 antibody, and thus, an anti-idiotype antibody mimics an epitope of Zwnt5.

An "antibody fragment" is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. For example, an anti-Zwnt5 monoclonal antibody fragment binds with an epitope of Zwnt5.

The term "antibody fragment" also includes a synthetic or a genetically engineered polypeptide that binds to a specific antigen, such as polypeptides consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

A "chimeric antibody" is a recombinant protein that contains the variable domains and complementary determining regions derived from a rodent antibody, while the remainder of the antibody molecule is derived from a human antibody.

"Humanized antibodies" are recombinant proteins in which murine complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, a "therapeutic agent" is a molecule or atom that is conjugated to an antibody moiety to produce a conjugate which is useful for therapy. Examples of therapeutic agents include drugs, toxins, immunomodulators, chelators, boron compounds, photoactive agents or dyes, and radioisotopes.

A "detectable label" is a molecule or atom that can be conjugated to an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable labels include chelators, photoactive agents, radioisotopes, fluorescent agents, paramagnetic ions, or other marker moieties.

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson *et al., EMBO J. 4*:1075 (1985); Nilsson *et al., Methods Enzymol. 198*:3 (1991)), glutathione S transferase (Smith and Johnson, *Gene 67*:31 (1988)), Glu-Glu affinity tag (Grussenmeyer *et al., Proc. Natl. Acad. Sci. USA 82*:7952 (1985)), substance P, FLAG peptide (Hopp *et al., Biotechnology 6*:1204 (1988)), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al., Protein Expression and Purification 2*:95 (1991). DNAs encoding affinity tags are available from commercial suppliers (*e.g.,* Pharmacia Biotech, Piscataway, NJ).

A "naked antibody" is an entire antibody, as opposed to an antibody fragment, which is not conjugated with a therapeutic agent. Naked antibodies include both polyclonal and monoclonal antibodies, as well as certain recombinant antibodies, such as chimeric and humanized antibodies.

As used herein, the term "antibody component" includes both an entire antibody and an antibody fragment.

An "immunoconjugate" is a conjugate of an antibody component with a therapeutic agent or a detectable label.

As used herein, the term "antibody fusion protein" refers to a recombinant molecule that comprises an antibody component and a therapeutic agent. Examples of therapeutic agents suitable for such fusion proteins include immunomodulators ("antibody-immunomodulator fusion protein") and toxins ("antibody-toxin fusion protein").

A "tumor associated antigen" is a protein normally not expressed, or expressed at lower levels, by a normal counterpart cell. Examples of tumor associated antigens include alpha-fetoprotein, carcinoembryonic antigen, and Her-2/neu. Many other illustrations of tumor-associated antigens are known. See, for example, Urban *et al., Ann. Rev. Immunol. 10*:617 (1992).

As used herein, an "infectious agent" denotes both microbes and parasites. A "microbe" includes viruses, bacteria, rickettsia, mycoplasma, protozoa, fungi and like microorganisms. A "parasite" denotes infectious, generally microscopic or very small multicellular invertebrates, or ova or juvenile forms thereof, which are susceptible to immune-mediated clearance or lytic or phagocytic destruction, such as malarial parasites, spirochetes, and the like.

An "infectious agent antigen" is an antigen associated with an infectious agent.

A "target polypeptide" or a "target peptide" is an amino acid sequence that comprises at least one epitope, and that is expressed on a target cell, such as a tumor cell, or a cell that carries an infectious agent antigen. T cells recognize peptide epitopes presented by a major histocompatibility complex molecule to a target polypeptide or target peptide and typically lyse the target cell or recruit other immune cells to the site of the target cell, thereby killing the target cell.

An "antigenic peptide" is a peptide that will bind a major histocompatibility complex molecule to form an MHC-peptide complex, which is recognized by a T cell, thereby inducing a cytotoxic lymphocyte response upon presentation to the T cell. Thus, antigenic peptides are capable of binding to an appropriate major histocompatibility complex molecule and inducing a cytotoxic T cells response, such as cell lysis or specific cytokine release against the target cell that binds or expresses the antigen. The antigenic peptide can be bound in the context of a class I or class II major histocompatibility complex molecule, on an antigen presenting cell or on a target cell.

In eukaryotes, RNA polymerase II catalyzes the transcription of a structural gene to produce mRNA. A nucleic acid molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The RNA transcript is termed an "anti-sense RNA" and a nucleic acid molecule that encodes the anti-sense RNA is termed an "anti-sense gene." Anti-sense RNA molecules are capable of binding to mRNA molecules, resulting in an inhibition of mRNA translation.

An "anti-sense oligonucleotide specific for Zwnt5" or a "Zwnt5 anti-sense oligonucleotide" is an oligonucleotide having a sequence (a) capable of forming a stable triplex with a portion of the *Zwnt5* gene, or (b) capable of forming a stable duplex with a portion of an mRNA transcript of the *Zwnt5* gene.

A "ribozyme" is a nucleic acid molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions. A nucleic acid molecule that encodes a ribozyme is termed a "ribozyme gene."

An "external guide sequence" is a nucleic acid molecule that directs the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, resulting in the cleavage of the mRNA by RNase P. A nucleic acid molecule that encodes an external guide sequence is termed an "external guide sequence gene."

The term "variant human *Zwnt5* gene" refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID NO: 2. Such variants include naturally-occurring polymorphisms of *Zwnt5* genes, as well as synthetic genes that contain conservative amino acid substitutions of the amino acid sequence of SEQ ID NOs: 2 or 3. Additional variant forms of *Zwnt5* genes are nucleic acid molecules that contain insertions or deletions of the nucleotide sequences described herein. A variant *Zwnt5* gene can be identified by determining whether the gene hybridizes with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1, or its complement, under stringent conditions.

Alternatively, variant *Zwnt5*genes can be identified by sequence comparison. Two amino acid sequences have "100% amino acid sequence identity" if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have "100% nucleotide sequence identity" if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art (see, for example, Peruski and Peruski, *The Internet and the New Biology: Tools for Genomic and Molecular Research* (ASM Press, Inc. 1997), Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," in *Methods in Gene Biotechnology*, pages 123-151 (CRC Press, Inc. 1997), and Bishop (ed.), *Guide to Human Genome Computing*, 2nd Edition (Academic Press, Inc. 1998)). Particular methods for determining sequence identity are described below.

Regardless of the particular method used to identify a variant *Zwnt5* gene or variant Zwnt5 polypeptide, a variant gene or polypeptide encoded by a variant gene is functionally characterized by either its anti-viral or anti-proliferative activities, or by the ability to bind specifically to an anti-Zwnt5 antibody.

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α-globin, β-globin, and myoglobin are paralogs of each other.

Due to the imprecision of standard analytical methods, molecular weights and lengths of polymers are understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

3. *Production of the Human Zwnt5 Gene*

Polynucleotides, generally a cDNA sequence, of the present invention encode the described polypeptides herein. A cDNA sequence which encodes a polypeptide of the present invention is comprised of a series of codons, each amino acid residue of the polypeptide being encoded by a codon and each codon being comprised of three nucleotides. The amino acid residues are encoded by their respective codons as follows.

Alanine (Ala) is encoded by GCA, GCC, GCG or GCT;
Cysteine (Cys) is encoded by TGC or TGT;
Aspartic acid (Asp) is encoded by GAC or GAT;
Glutamic acid (Glu) is encoded by GAA or GAG;
Phenylalanine (Phe) is encoded by TTC or TTT;
Glycine (Gly) is encoded by GGA, GGC, GGG or GGT;
Histidine (His) is encoded by CAC or CAT;
Isoleucine (Ile) is encoded by ATA, ATC or ATT;
Lysine (Lys) is encoded by AAA, or AAG;
Leucine (Leu) is encoded by TTA, TTG, CTA, CTC, CTG or CTT;
Methionine (Met) is encoded by ATG;
Asparagine (Asn) is encoded by AAC or AAT;
Proline (Pro) is encoded by CCA, CCC, CCG or CCT;
Glutamine (Gln) is encoded by CAA or CAG;

Arginine (Arg) is encoded by AGA, AGG, CGA, CGC, CGG or CGT;
Serine (Ser) is encoded by AGC, AGT, TCA, TCC, TCG or TCT;
Threonine (Thr) is encoded by ACA, ACC, ACG or ACT;
Valine (Val) is encoded by GTA, GTC, GTG or GTT;
Tryptophan (Trp) is encoded by TGG; and
Tyrosine (Tyr) is encoded by TAC or TAT.

It is to be recognized that according to the present invention, when a polynucleotide is claimed as described herein, it is understood that what is claimed are both the sense strand, the anti-sense strand, and the DNA as double-stranded having both the sense and anti-sense strand annealed together by their respective hydrogen bonds. Also claimed is the messenger RNA (mRNA) that encodes the polypeptides of the president invention, and which mRNA is encoded by the cDNA described herein. Messenger RNA (mRNA) will encode a polypeptide using the same codons as those defined herein, with the exception that each thymine nucleotide (T) is replaced by a uracil nucleotide (U).

Nucleic acid molecules encoding a human *Zwnt5*gene can be obtained by screening a human cDNA or genomic library using polynucleotide probes based upon SEQ ID NO: 1. These techniques are standard and well established.

As an illustration, a nucleic acid molecule that encodes a human *Zwnt5*gene can be isolated from a human cDNA library. In this case, the first step would be to prepare the cDNA library by isolating RNA mammary epithelial tissue. In general, RNA isolation techniques must provide a method for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and separating RNA from the remaining impurities by selective precipitation with lithium chloride [see, for example, Ausubel *et al.* (eds.), *Short Protocols in Molecular Biology, 3rd Edition*, pages 4-1 to 4-6 (John Wiley & Sons 1995)] ["Ausubel (1995)"]; Wu *et al.*, *Methods in Gene Biotechnology*, pages 33-41 (CRC Press, Inc. 1997) "Wu (1997)".

Alternatively, total RNA can be isolated from mammary epithelial tissue by extracting ground tissue with guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example,

Chirgwin *et al., Biochemistry 18*:52 (1979); Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

In order to construct a cDNA library, poly(A)$^+$ RNA must be isolated from a total RNA preparation. Poly(A)$^+$ RNA can be isolated from total RNA using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Aviv and Leder, *Proc. Nat'l Acad. Sci. USA 69:*1408 (1972); Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A)$^+$ RNA using techniques well-known to those in the art. (see, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life Technologies, Inc. (Gaithersburg, MD), CLONTECH Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI) and STRATAGENE (La Jolla, CA).

Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from bacteriophage, such as a λgt10 vector. See, for example, Huynh *et al.*, "Constructing and Screening cDNA Libraries in λgt10 and λgt11," in *DNA Cloning: A Practical Approach Vol. I*, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52.

Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a PBLUESCRIPT vector (STRATAGENE; La Jolla, CA), a LAMDAGEM-4 (Promega Corp.) or other commercially available vectors. Suitable cloning vectors also can be obtained from the American Type Culture Collection (Manassas, VA).

To amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library can be introduced into competent *E. coli* DH5 cells, which can be obtained, for example, from Life Technologies, Inc. (Gaithersburg, MD).

A human genomic library can be prepared by means well known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327). Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic DNA with restriction endonucleases. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini,

the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for such manipulation are well known in the art [see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327].

Nucleic acid molecules that encode a human *Zwnt5* gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the human *Zwnt5* gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Manassas, VA).

A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO: 1, using standard methods [see, for example, Ausubel (1995) at pages 6-1 to 6-11].

Anti-Zwnt5 antibodies, produced as described below, can also be used to isolate DNA sequences that encode human *Zwnt5*genes from cDNA libraries. For example, the antibodies can be used to screen λgt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation (see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis *et al.*, "Screening λ expression libraries with antibody and protein probes," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995)).

As an alternative, a *Zwnt5*gene can be obtained by synthesizing nucleic acid molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang *et al.*, *Plant Molec. Biol. 21*:1131 (1993), Bambot *et al.*, *PCR Methods and Applications 2*:266 (1993), Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current*

Methods and Applications, White (ed.), pages 263-268, (Humana Press, Inc. 1993), and Holowachuk *et al., PCR Methods Appl. 4*:299 (1995)).

The nucleic acid molecules of the present invention can also be synthesized with "gene machines" using protocols such as the phosphoramidite method. If chemically synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made separately. The production of short genes (60 to 80 base pairs) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer genes (>300 base pairs), however, special strategies may be required, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length.

One method for building a synthetic gene requires the initial production of a set of overlapping, complementary oligonucleotides, each of which is between 20 to 60 nucleotides long. The sequences of the strands are planned so that, after annealing, the two end segments of the gene are aligned to give blunt ends. Each internal section of the gene has complementary 3' and 5' terminal extensions that are designed to base pair precisely with an adjacent section. Thus, after the gene is assembled, the only remaining requirement to complete the process is to seal the nicks along the backbones of the two strands with T4 DNA ligase. In addition to the protein coding sequence, synthetic genes can be designed with terminal sequences that facilitate insertion into a restriction endonuclease sites of a cloning vector and other sequences should also be added that contain signals for the proper initiation and termination of transcription and translation.

An alternative way to prepare a full-size gene is to synthesize a specified set of overlapping oligonucleotides (40 to 100 nucleotides). After the 3' and 5' extensions (6 to 10 nucleotides) are annealed, large gaps still remain, but the base-paired regions are both long enough and stable enough to hold the structure together. The duplex is completed and the gaps filled by enzymatic DNA synthesis with *E. coli* DNA polymerase I. This enzyme uses the 3'-hydroxyl groups as replication initiation points and the single-stranded regions as templates. After the enzymatic synthesis is completed, the nicks are sealed with T4 DNA ligase. For larger genes, the complete gene sequence is usually assembled from double-stranded fragments that are each put together by joining four to six overlapping oligonucleotides (20 to 60 base pairs each). If there is a sufficient amount of the double-stranded fragments after each synthesis and annealing step, they are simply joined to one another. Otherwise, each fragment is cloned into a vector to amplify the amount of DNA available. In both cases, the double-stranded

constructs are sequentially linked to one another to form the entire gene sequence. Each double-stranded fragment and the complete sequence should be characterized by DNA sequence analysis to verify that the chemically synthesized gene has the correct nucleotide sequence. For reviews on polynucleotide synthesis, see, for example, Glick and Pasternak, *Molecular Biotechnology, Principles and Applications of Recombinant DNA* (ASM Press 1994), Itakura *et al., Annu. Rev. Biochem. 53*:323 (1984), and Climie *et al., Proc. Nat'l Acad. Sci. USA 87*:633 (1990).

The sequence of a *Zwnt5*cDNA or *Zwnt5* genomic fragment can be determined using standard methods. Moreover, the identification of genomic fragments containing a *Zwnt5ε* promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

Cloning of 5' flanking sequences also facilitates production of Zwnt5 proteins by "gene activation," following the methods disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous *Zwnt5*gene in a cell is altered by introducing into the *Zwnt5*locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a *Zwnt5* 5' non-coding sequence that permits homologous recombination of the construct with the endogenous *Zwnt5* locus, whereby the sequences within the construct become operably linked with the endogenous *Zwnt5* coding sequence. In this way, an endogenous *Zwnt5*promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

4. Production of Zsig9 Gene Variants

The present invention provides a variety of nucleic acid molecules, including DNA and RNA molecules, that encode the Zwnt5 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. Table 1 sets forth the one-letter codes used within SEQ ID NOs:3, 6, and 25 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

Table 1

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NOs:3, 6, and 25, encompassing all possible codons for a given amino acid, are set forth in Table 2.

Table 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding an amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 3. Variant sequences can be readily tested for functionality as described herein.

Different species can exhibit "preferential codon usage." In general, see, Grantham *et al., Nuc. Acids Res. 8*:1893 (1980), Haas *et al. Curr. Biol. 6*:315 (1996), Wain-Hobson *et al., Gene 13*:355 (1981), Grosjean and Fiers, *Gene 18*:199 (1982), Holm, *Nuc. Acids Res. 14*:3075 (1986), Ikemura, *J. Mol. Biol. 158*:573 (1982), Sharp and Matassi, *Curr. Opin. Genet. Dev. 4*:851 (1994), Kane, *Curr. Opin. Biotechnol. 6*:494 (1995), and Makrides, *Microbiol. Rev. 60*:512 (1996). As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid Threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species

The present invention further provides variant polypeptides and nucleic acid molecules that represent counterparts from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are Zwnt5 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human Zwnt5 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses Zwnt5ε as disclosed herein. Suitable sources of mRNA can be identified by probing northern

blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line.

A Zwnt5-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction with primers designed from the representative human Zwnt5 sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to Zwnt5 polypeptide. Similar techniques can also be applied to the isolation of genomic clones, and to the isolation of nucleic molecules that encode murine Zwnt5.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO: 1 represents a single allele of human Zwnt5, and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the nucleotide sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NOs: 2 and 3. cDNA molecules generated from alternatively spliced mRNAs, which retain the properties of the Zwnt5 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

Within preferred embodiments of the invention, isolated nucleic acid molecules that encode human Zwnt5 can hybridize to nucleic acid molecules having the nucleotide sequence of SEQ ID NO: 1, or a sequence complementary thereto, under "stringent conditions." In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

As an illustration, a nucleic acid molecule encoding a variant Zwnt5 polypeptide can be hybridized with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) at 42°C overnight in a solution comprising 50% formamide, 5xSSC (1xSSC: 0.15 M sodium chloride and 15 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution (100x Denhardt's solution: 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, and 2%

(w/v) bovine serum albumin), 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA. One of skill in the art can devise variations of these hybridization conditions. For example, the hybridization mixture can be incubated at a higher temperature, such as about 65°C, in a solution that does not contain formamide. Moreover, premixed hybridization solutions are available (*e.g.*, EXPRESSHYB Hybridization Solution from CLONTECH Laboratories, Inc.), and hybridization can be performed according to the manufacturer's instructions.

Following hybridization, the nucleic acid molecules can be washed to remove non-hybridized nucleic acid molecules under stringent conditions, or under highly stringent conditions. Typical stringent washing conditions include washing in a solution of 0.5x - 2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 55 - 65°C. That is, nucleic acid molecules encoding a variant Zwnt5ε polypeptide hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, including 0.5x SSC with 0.1% SDS at 55°C, or 2xSSC with 0.1% SDS at 65°C. One of skill in the art can readily devise equivalent conditions, for example, by substituting SSPE for SSC in the wash solution.

Typical highly stringent washing conditions include washing in a solution of 0.1x - 0.2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 50 - 65°C. In other words, nucleic acid molecules encoding a variant Zwnt5 polypeptide hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, including 0.1x SSC with 0.1% SDS at 50°C, or 0.2xSSC with 0.1% SDS at 65°C.

The present invention also provides isolated Zwnt5 polypeptides that have a substantially similar sequence identity to the polypeptides of SEQ ID NO: 2, SEQ ID NO:3, or their orthologs. The term "substantially similar sequence identity" is used herein to denote polypeptides having at least at least 90%, at least 95% or greater sequence identity to the sequences shown in SEQ ID NO: 2, SEQ ID NO: 3, or their orthologs.

The present invention also contemplates Zwnt5 variant nucleic acid molecules that can be identified using two criteria: a determination of the similarity between the encoded polypeptide with the amino acid sequence of SEQ ID NO:2, and a hybridization assay, as described above. Such Zwnt5 variants include nucleic acid molecules (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under stringent washing conditions, in

which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, and (2) that encode a polypeptide having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NO:2. Alternatively, Zwnt5ε variants can be characterized as nucleic acid molecules (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, and (2) that encode a polypeptide having at least 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NO:2.

The present invention also contemplates human Zwnt5 variant nucleic acid molecules identified by at least one of hybridization analysis and sequence identity determination, with reference to SEQ ID NOs: 1 and 2.

Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al., Bull. Math. Bio. 48*:603 (1986), and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA 89*:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM 62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as: ([Total number of identical matches]/ [length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences])(100).

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative Zwnt5 variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA 85*:2444 (1988), and by Pearson, *Meth. Enzymol. 183*:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then re-scored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol. 48*:444 (1970); Sellers, *SIAM J. Appl. Math. 26*:787 (1974)), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol. 183*:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as described above.

The present invention includes nucleic acid molecules that encode a polypeptide having a conservative amino acid change, compared with the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NO: 2 or SEQ ID NO: 3, in which an alkyl amino acid is substituted for an alkyl amino acid in an Zwnt5 amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in an Zwnt5 amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in an Zwnt5 amino acid sequence, a hydroxy-containing amino

acid is substituted for a hydroxy-containing amino acid in an Zwnt5 amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in an Zwnt5 amino acid sequence, a basic amino acid is substituted for a basic amino acid in an Zwnt5 amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in an Zwnt5 amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine.

The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA 89*:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed above), the language "conservative amino acid substitution" preferably refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (*e.g.*, 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (*e.g.*, 2 or 3).

Conservative amino acid changes in an *Zwnt5* gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NOs:1. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)). The ability of such variants to promote anti-viral or anti-proliferative activity can be determined using a standard method, such as the assay described herein. Alternatively, a variant Zwnt5 polypeptide can be identified by the ability to specifically bind anti-Zwnt5 antibodies.

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine,

hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is typically carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson *et al., J. Am. Chem. Soc. 113*:2722 (1991), Ellman *et al., Methods Enzymol. 202*:301 (1991), Chung *et al., Science 259*:806 (1993), and Chung *et al., Proc. Nat'l Acad. Sci. USA 90*:10145 (1993).

In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs [Turcatti *et al., J. Biol. Chem. 271*:19991 (1996)]. Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (*e.g.*, phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (*e.g.*, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al., Biochem. 33*:7470 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci. 2*:395 (1993)).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zwnt5 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science 244*:1081 (1989), Bass *et al., Proc. Nat'l Acad. Sci. USA 88*:4498 (1991), Coombs and Corey, "Site-Directed Mutagenesis and Protein Engineering," in *Proteins: Analysis and Design*, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid

residues that are critical to the activity of the molecule. See also, Hilton *et al., J. Biol. Chem. 271*:4699 (1996).

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer [*Science 241*:53 (1988)] or Bowie and Sauer [*Proc. Nat'l Acad. Sci. USA 86*:2152 (1989)]. Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display [*e.g.*, Lowman *et al., Biochem. 30*:10832 (1991), Ladner *et al.*, U.S. Patent No. 5,223,409, Huse, international publication No. WO 92/06204, and region-directed mutagenesis (Derbyshire *et al., Gene 46*:145 (1986), and Ner *et al., DNA 7*:127, (1988)].

Variants of the disclosed Zwnt5nucleotide and polypeptide sequences can also be generated through DNA shuffling as disclosed by Stemmer, *Nature 370*:389 (1994), Stemmer, *Proc. Nat'l Acad. Sci. USA 91*:10747 (1994), and international publication No. WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active polypeptides, or polypeptides that bind with anti-Zwnt5 antibodies, can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

The present invention also includes "functional fragments" of Zwnt5 polypeptides and nucleic acid molecules encoding such functional fragments. Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a Zwnt5 polypeptide.

The present invention also provides polypeptide fragments or peptides comprising an epitope-bearing portion of a Zwnt5 polypeptide described herein. Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a

protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods (see, for example, Geysen *et al., Proc. Nat'l Acad. Sci. USA 81*:3998 (1983)).

In contrast, polypeptide fragments or peptides may comprise an "antigenic epitope," which is a region of a protein molecule to which an antibody can specifically bind. Certain epitopes consist of a linear or contiguous stretch of amino acids, and the antigenicity of such an epitope is not disrupted by denaturing agents. It is known in the art that relatively short synthetic peptides that can mimic epitopes of a protein can be used to stimulate the production of antibodies against the protein (see, for example, Sutcliffe *et al., Science 219*:660 (1983)). Accordingly, antigenic epitope-bearing peptides and polypeptides of the present invention are useful to raise antibodies that bind with the polypeptides described herein.

Antigenic epitope-bearing peptides and polypeptides preferably contain at least four to ten amino acids, at least ten to fifteen amino acids, or about 15 to about 30 amino acids of SEQ ID NO: 2 or SEQ ID NO: 3. Such epitope-bearing peptides and polypeptides can be produced by fragmenting a Zwnt5 polypeptide, or by chemical peptide synthesis, as described herein. Moreover, epitopes can be selected by phage display of random peptide libraries (see, for example, Lane and Stephen, *Curr. Opin. Immunol. 5*:268 (1993), and Cortese *et al., Curr. Opin. Biotechnol. 7*:616 (1996)). Standard methods for identifying epitopes and producing antibodies from small peptides that comprise an epitope are described, for example, by Mole, "Epitope Mapping," in *Methods in Molecular Biology, Vol. 10*, Manson (ed.), pages 105-116 (The Humana Press, Inc. 1992), Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 60-84 (Cambridge University Press 1995), and Coligan *et al.* (eds.), *Current Protocols in Immunology*, pages 9.3.1 - 9.3.5 and pages 9.4.1 - 9.4.11 (John Wiley & Sons 1997). Examples of such epitope-bearing polypeptides are polypeptides comprised of SEQ ID NOs: 4-20.

For any Zwnt5 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above. Moreover, those of skill in the art can use standard software to devise Zwnt5 variants based upon the nucleotide and amino acid sequences described herein. Accordingly, the present invention includes a computer-readable medium encoded with a data structure that provides at least one of the following sequences: SEQ ID NOs: 1-20. Suitable forms of computer-readable media include magnetic media and optically-readable media. Examples of magnetic media include a hard or fixed drive, a random access memory

(RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP disk. Optically readable media are exemplified by compact discs (*e.g.*, CD-read only memory (ROM), CD-rewritable (RW), and CD-recordable), and digital versatile/video discs (DVD) (*e.g.*, DVD-ROM, DVD-RAM, and DVD+RW).

5

5. Production of Zwnt5 Fusion Proteins and Conjugates

Fusion proteins of Zwnt5 can be used to express Zwnt5 in a recombinant host, and to isolate expressed Zwnt5. As described below, particular Zwnt5 fusion proteins also have uses in diagnosis and therapy.

10 One type of fusion protein comprises a peptide that guides a Zwnt5 polypeptide from a recombinant host cell. To direct a Zwnt5 polypeptide into the secretory pathway of a eukaryotic host cell, a secretory signal sequence (also known as a signal peptide, a leader sequence, prepro sequence or pre sequence) is provided in the Zwnt5 expression vector. While the secretory signal sequence may be derived from 15 Zwnt5, a suitable signal sequence may also be derived from another secreted protein or synthesized *de novo*. The secretory signal sequence is operably linked to a Zwnt5-encoding sequence such that the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleotide 20 sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleotide sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

Although the secretory signal sequence of Zwnt5 or another protein produced by mammalian cells (*e.g.*, tissue-type plasminogen activator signal sequence, 25 as described, for example, in U.S. Patent No. 5,641,655) is useful for expression of Zwnt5 in recombinant mammalian hosts, a yeast signal sequence is preferred for expression in yeast cells. Examples of suitable yeast signal sequences are those derived from yeast mating phermone α-factor (encoded by the *MFα1* gene), invertase (encoded by the *SUC2* gene), or acid phosphatase (encoded by the *PHO5* gene). See, for example, 30 Romanos *et al.*, "Expression of Cloned Genes in Yeast," in *DNA Cloning 2: A Practical Approach*, 2nd Edition, Glover and Hames (eds.), pages 123-167 (Oxford University Press 1995).

In bacterial cells, it is often desirable to express a heterologous protein as a fusion protein to decrease toxicity, increase stability, and to enhance recovery of the 35 expressed protein. For example, Zwnt5 can be expressed as a fusion protein comprising a glutathione S-transferase polypeptide. Glutathione S-transferease fusion proteins are

typically soluble, and easily purifiable from *E. coli* lysates on immobilized glutathione columns. In similar approaches, a Zwnt5 fusion protein comprising a maltose binding protein polypeptide can be isolated with an amylose resin column, while a fusion protein comprising the C-terminal end of a truncated Protein A gene can be purified using IgG-Sepharose. Established techniques for expressing a heterologous polypeptide as a fusion protein in a bacterial cell are described, for example, by Williams *et al.*, "Expression of Foreign Proteins in *E. coli* Using Plasmid Vectors and Purification of Specific Polyclonal Antibodies," in *DNA Cloning 2: A Practical Approach,* 2nd Edition, Glover and Hames (Eds.), pages 15-58 (Oxford University Press 1995). In addition, commercially available expression systems are available. For example, the PINPOINT Xa protein purification system (Promega Corporation; Madison, WI) provides a method for isolating a fusion protein comprising a polypeptide that becomes biotinylated during expression with a resin that comprises avidin.

Peptide tags that are useful for isolating heterologous polypeptides expressed by either prokaryotic or eukaryotic cells include polyHistidine tags (which have an affinity for nickel-chelating resin), *c-myc* tags, calmodulin binding protein (isolated with calmodulin affinity chromatography), substance P, the RYIRS tag (which binds with anti-RYIRS antibodies), the Glu-Glu tag, and the FLAG tag (which binds with anti-FLAG antibodies). See, for example, Luo *et al., Arch. Biochem. Biophys. 329*:215 (1996), Morganti *et al., Biotechnol. Appl. Biochem. 23*:67 (1996), and Zheng *et al., Gene 186*:55 (1997). Nucleic acid molecules encoding such peptide tags are available, for example, from Sigma-Aldrich Corporation (St. Louis, MO).

The present invention also contemplates that the use of the secretory signal sequence contained in the Zwnt5 polypeptides of the present invention to direct other polypeptides into the secretory pathway. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from amino acid residues 1 to 27 of SEQ ID NO:2 is operably linked to another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein, such as a receptor. Such fusions may be used in a transgenic animal or in a cultured recombinant host to direct peptides through the secretory pathway. With regard to the latter, exemplary polypeptides include pharmaceutically active molecules such as Factor VIIa, proinsulin, insulin, follicle stimulating hormone, tissue type plasminogen activator, tumor necrosis factor, interleukins (*e.g.*, interleukin-1 (IL-1), IL-2, IL-3, IL-4,

IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15), colony stimulating factors (*e.g.*, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)), interferons (*e.g.*, interferons-α, -β, -γ, -ω, -δ, and -τ), the stem cell growth factor designated "S1 factor," erythropoietin, and thrombopoietin. The Zwnt5 secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Fusion proteins comprising a Zwnt5 secretory signal sequence can be constructed using standard techniques.

Another form of fusion protein comprises a Zwnt5 polypeptide and an immunoglobulin heavy chain constant region, typically an F_C fragment, which contains two or three constant region domains and a hinge region but lacks the variable region. As an illustration, Chang *et al.*, U.S. Patent No. 5,723,125, describe a fusion protein comprising a human interferon and a human immunoglobulin Fc fragment. The C-terminal of the interferon is linked to the N-terminal of the Fc fragment by a peptide linker moiety. An example of a peptide linker is a peptide comprising primarily a T cell inert sequence, which is immunologically inert. An exemplary peptide linker has the amino acid sequence: GGSGG SGGGG SGGGG S (SEQ ID NO:21). In this fusion protein, a preferred Fc moiety is a human γ4 chain, which is stable in solution and has little or no complement activating activity. Accordingly, the present invention contemplates an Zwnt5 fusion protein that comprises an Zwnt5 moiety and a human Fc fragment, wherein the C-terminus of the Zwnt5ε moiety is attached to the N-terminus of the Fc fragment via a peptide linker, such as a peptide consisting of the amino acid sequence of SEQ ID NO: 21. The Zwnt5 moiety can be a Zwnt5 molecule or a fragment thereof.

In another variation, an Zwnt5 fusion protein comprises an IgG sequence, an Zwnt5 moiety covalently joined to the aminoterminal end of the IgG sequence, and a signal peptide that is covalently joined to the aminoterminal of the Zwnt5 moiety, wherein the IgG sequence consists of the following elements in the following order: a hinge region, a CH_2 domain, and a CH_3 domain. Accordingly, the IgG sequence lacks a CH_1 domain. This general approach to producing fusion proteins that comprise both antibody and nonantibody portions has been described by LaRochelle *et al.*, EP 742830 (WO 95/21258).

Fusion proteins comprising a Zwnt5 moiety and an Fc moiety can be used, for example, as an *in vitro* assay tool. For example, the presence of a Zwnt5 receptor in a biological sample can be detected using a Zwnt5-immunoglobulin fusion protein, in which the Zwnt5 moiety is used to target the cognate receptor, and a

macromolecule, such as Protein A or anti-Fc antibody, is used to detect the bound fusion protein-receptor complex. Moreover, such fusion proteins can be used to identify agonists and antagonists that interfere with the binding of Zwnt5 to its receptor.

In addition, antibody-Zwnt5 fusion proteins, comprising antibody variable domains, are useful as therapeutic proteins, in which the antibody moiety binds with a target antigen, such as a tumor associated antigen. Methods of making antibody-cytokine fusion proteins are known to those of skill in the art. For example, antibody fusion proteins comprising an interleukin-2 moiety are described by Boleti *et al., Ann. Oncol. 6*:945 (1995), Nicolet *et al., Cancer Gene Ther. 2*:161 (1995), Becker *et al., Proc. Nat'l Acad. Sci. USA 93*:7826 (1996), Hank *et al., Clin. Cancer Res. 2*:1951 (1996), and Hu *et al., Cancer Res. 56*:4998 (1996). Moreover, Yang *et al., Hum. Antibodies Hybridomas 6*:129 (1995), and Xiang *et al., J. Biotechnol. 53*:3 (1997), describe fusion proteins that include an $F(ab')_2$ fragment and a tumor necrosis factor alpha moiety. Additional cytokine-antibody fusion proteins include IL-8, IL-12, or Zwnt5as the cytokine moiety (Holzer *et al., Cytokine 8*:214 (1996); Gillies *et al., J. Immunol. 160*:6195 (1998); Xiang *et al., Hum. Antibodies Hybridomas 7*:2 (1996)). Also see, Gillies, U.S. Patent No. 5,650,150.

Moreover, using methods described in the art, hybrid Zwnt5 proteins can be constructed using regions or domains of the inventive (see, for example, Picard, *Cur. Opin. Biology 5*:511 (1994)). These methods allow the determination of the biological importance of larger domains or regions in a polypeptide of interest. Such hybrids may alter reaction kinetics, binding, constrict or expand the substrate specificity, or alter tissue and cellular localization of a polypeptide, and can be applied to polypeptides of unknown structure. Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding both components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. Moreover, such fusion proteins may exhibit other properties as disclosed herein. General methods for enzymatic and chemical cleavage of fusion proteins are described, for example, by Ausubel (1995) at pages 16-19 to 16-25.

The present invention also contemplates chemically modified Zwnt5 compositions, in which a Zwnt5 polypeptide is linked with a polymer. Typically, the polymer is water-soluble so that the Zwnt5 conjugate does not precipitate in an aqueous environment, such as a physiological environment. An example of a suitable polymer is one that has been modified to have a single reactive group, such as an active ester for acylation, or an aldehyde for alkylation. In this way, the degree of polymerization can be controlled. An example of a reactive aldehyde is polyethylene glycol propionaldehyde, or mono-(C1-C10) alkoxy, or aryloxy derivatives thereof (see, for example, Harris, *et al.*,

U.S. Patent No. 5,252,714). The polymer may be branched or unbranched. Moreover, a mixture of polymers can be used to produce Zwnt5 conjugates.

Zwnt5 conjugates used for therapy should preferably comprise pharmaceutically acceptable water-soluble polymer moieties. Conjugation of interferons with water-soluble polymers has been shown to enhance the circulating half-life of the interferon, and to reduce the immunogenicity of the polypeptide [see, for example, Nieforth *et al., Clin. Pharmacol. Ther. 59*:636 (1996), and Monkarsh *et al., Anal. Biochem. 247*:434 (1997)].

Suitable water-soluble polymers include polyethylene glycol (PEG), monomethoxy-PEG, mono-(C1-C10)alkoxy-PEG, aryloxy-PEG, poly-(N-vinyl pyrrolidone)PEG, tresyl monomethoxy PEG, PEG propionaldehyde, *bis*-succinimidyl carbonate PEG, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, dextran, cellulose, or other carbohydrate-based polymers. Suitable PEG may have a molecular weight from about 600 to about 60,000, including, for example, 5,000, 12,000, 20,000 and 25,000. A Zwnt5 conjugate can also comprise a mixture of such water-soluble polymers.

One example of a Zwnt5 conjugate comprises a Zwnt5 moiety and a polyalkyl oxide moiety attached to the *N*-terminus of the Zwnt5 moiety. PEG is one suitable polyalkyl oxide. As an illustration, Zwnt5 can be modified with PEG, a process known as "PEGylation." PEGylation of Zwnt5 can be carried out by any of the PEGylation reactions known in the art [see, for example, EP 0 154 316, Delgado *et al., Critical Reviews in Therapeutic Drug Carrier Systems 9*:249 (1992), Duncan and Spreafico, *Clin. Pharmacokinet. 27*:290 (1994), and Francis *et al., Int J Hematol 68*:1 (1998)]. For example, PEGylation can be performed by an acylation reaction or by an alkylation reaction with a reactive polyethylene glycol molecule. In an alternative approach, Zwnt5 conjugates are formed by condensing activated PEG, in which a terminal hydroxy or amino group of PEG has been replaced by an activated linker (see, for example, Karasiewicz *et al.*, U.S. Patent No. 5,382,657).

PEGylation by acylation typically requires reacting an active ester derivative of PEG with a Zwnt5 polypeptide. An example of an activated PEG ester is PEG esterified to *N*-hydroxysuccinimide. As used herein, the term "acylation" includes the following types of linkages between Zwnt5 and a water-soluble polymer: amide, carbamate, urethane, and the like. Methods for preparing PEGylated Zwnt5 by acylation will typically comprise the steps of (a) reacting an Zwnt5 polypeptide with PEG (such as a reactive ester of an aldehyde derivative of PEG) under conditions whereby one or more PEG groups attach to Zwnt5, and (b) obtaining the reaction product(s). Generally, the

optimal reaction conditions for acylation reactions will be determined based upon known parameters and desired results. For example, the larger the ratio of PEG: Zwnt5, the greater the percentage of polyPEGylated Zwnt5 product.

The product of PEGylation by acylation is typically a polyPEGylated Zwnt5 product, wherein the lysine ε-amino groups are PEGylated via an acyl linking group. An example of a connecting linkage is an amide. Typically, the resulting Zwnt5 will be at least 95% mono-, di-, or tri-pegylated, although some species with higher degrees of PEGylation may be formed depending upon the reaction conditions. PEGylated species can be separated from unconjugated Zwnt5 polypeptides using standard purification methods, such as dialysis, ultrafiltration, ion exchange chromatography, affinity chromatography, and the like.

PEGylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with Zwnt5 in the presence of a reducing agent. PEG groups are preferably attached to the polypeptide via a $-CH_2-NH$ group.

Derivatization via reductive alkylation to produce a monoPEGylated product takes advantage of the differential reactivity of different types of primary amino groups available for derivatization. Typically, the reaction is performed at a pH that allows one to take advantage of the pKa differences between the ε-amino groups of the lysine residues and the α-amino group of the *N*-terminal residue of the protein. By such selective derivatization, attachment of a water-soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled. The conjugation with the polymer occurs predominantly at the *N*-terminus of the protein without significant modification of other reactive groups such as the lysine side chain amino groups. The present invention provides a substantially homogenous preparation of Zwnt5 monopolymer conjugates.

Reductive alkylation to produce a substantially homogenous population of monopolymer Zwnt5 conjugate molecule can comprise the steps of: (a) reacting an Zwnt5 polypeptide with a reactive PEG under reductive alkylation conditions at a pH suitable to permit selective modification of the α-amino group at the amino terminus of the Zwnt5, and (b) obtaining the reaction product(s). The reducing agent used for reductive alkylation should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents include sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane, and pyridine borane.

For a substantially homogenous population of monopolymer Zwnt5 conjugates, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the *N*-terminus of Zwnt5. Such reaction conditions generally provide for pKa differences between the lysine amino

groups and the α-amino group at the *N*-terminus. The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired because the less reactive the *N*-terminal α-group, the more polymer is needed to achieve optimal conditions. If the pH is higher, the polymer:Zwnt5 need not be as large because more reactive groups are available. Typically, the pH will fall within the range of 3 - 9, or 3 - 6.

Another factor to consider is the molecular weight of the water-soluble polymer. Generally, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. For PEGylation reactions, the typical molecular weight is about 2 kDa to about 100 kDa, about 5 kDa to about 50 kDa, or about 12 kDa to about 25 kDa. The molar ratio of water-soluble polymer to Zwnt5 will generally be in the range of 1:1 to 100:1. Typically, the molar ratio of water-soluble polymer to Zwnt5 will be 1:1 to 20:1 for polyPEGylation, and 1:1 to 5:1 for monoPEGylation.

General methods for producing conjugates comprising interferon and water-soluble polymer moieties are known in the art. See, for example, Karasiewicz *et al.*, U.S. Patent No. 5,382,657, Greenwald *et al.*, U.S. Patent No. 5,738, 846, Nieforth *et al., Clin. Pharmacol. Ther. 59*:636 (1996), Monkarsh *et al., Anal. Biochem. 247*:434 (1997).

6. Production of Zwnt5 Polypeptides in Cultured Cells

The polypeptides of the present invention, including full-length polypeptides, functional fragments, and fusion proteins, can be produced in recombinant host cells following conventional techniques. To express a *Zwnt5* gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that control transcriptional expression in an expression vector and then, introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence. As discussed above, expression vectors can also include nucleotide sequences

encoding a secretory sequence that directs the heterologous polypeptide into the secretory pathway of a host cell. For example, a Zwnt5 expression vector may comprise a *Zwnt5* gene and a secretory sequence derived from a *Zwnt5* gene or another secreted gene.

Zwnt5 proteins of the present invention may be expressed in mammalian cells. Examples of suitable mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21, BHK-570; ATCC CRL 8544, ATCC CRL 10314), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61; CHO DG44 [Chasin *et al., Som. Cell. Molec. Genet. 12*:555 (1986)]], rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as *actin*, *collagen*, *myosin*, and *metallothionein* genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse *metallothionein I* gene [Hamer *et al., J. Molec. Appl. Genet. 1*:273 (1982)], the *TK* promoter of *Herpes* virus [McKnight, *Cell 31*:355 (1982)], the *SV40* early promoter [Benoist *et al., Nature 290*:304 (1981)], the *Rous* sarcoma virus promoter (Gorman *et al., Proc. Nat'l Acad. Sci. USA 79*:6777 (1982)), the cytomegalovirus promoter [Foecking *et al., Gene 45*:101 (1980)], and the mouse mammary tumor virus promoter [see, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)].

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control *Zwnt5* gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter [Zhou *et al., Mol. Cell. Biol. 10*:4529 (1990), and Kaufman *et al., Nucl. Acids Res. 19*:4485 (1991)].

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. Preferably, the transfected cells are selected and propagated to provide recombinant host cells that

comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), *Gene Transfer and Expression Protocols* (Humana Press 1991).

For example, one suitable selectable marker is a gene that provides resistance to the antibiotic neomycin. In this case, selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g.*, hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternatively, markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Zwnt5 polypeptides can also be produced by cultured mammalian cells using a viral delivery system. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid [for a review, see Becker *et al., Meth. Cell Biol. 43*:161 (1994), and Douglas and Curiel, *Science & Medicine 4*:44 (1997)]. Advantages of the adenovirus system include the accommodation of relatively large DNA inserts, the ability to grow to high-titer, the ability to infect a broad range of mammalian cell types, and flexibility that allows use with a large number of available vectors containing different promoters.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. An option is to delete the essential *E1* gene from the viral vector, which results in the inability to replicate unless the *E1* gene is provided by the host cell. Adenovirus vector-infected human 293 cells (ATCC Nos. CRL-1573, 45504, 45505), for example, can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein [see Garnier *et al., Cytotechnol. 15*:145 (1994)].

Zwnt5 genes may also be expressed in other higher eukaryotic cells, such as avian, fungal, insect, yeast, or plant cells. The baculovirus system provides an efficient means to introduce cloned Zwnt5 genes into insect cells. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila heat shock protein* (*hsp*) 70 promoter, *Autographa californica nuclear polyhedrosis virus immediate-early* gene promoter (*ie-1*) and the *delayed early 39K* promoter, baculovirus *p10* promoter, and the *Drosophila metallothionein* promoter. A second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, *et al., J. Virol. 67*:4566 (1993)). This system, which utilizes transfer vectors, is sold in the BAC-to-BAC kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, PFASTBAC (Life Technologies) containing a Tn7 transposon to move the DNA encoding the Zwnt5ε polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol. 71*:971 (1990), Bonning, *et al., J. Gen. Virol. 75*:1551 (1994), and Chazenbalk, and Rapoport, *J. Biol. Chem. 270*:1543 (1995). In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed Zwnt5 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer *et al., Proc. Nat'l Acad. Sci. 82*:7952 (1985)). Using a technique known in the art, a transfer vector containing a *Zwnt5* gene is transformed into *E. coli*, and screened for bacmids that contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is then isolated using common techniques.

The illustrative PFASTBAC vector can be modified to a considerable degree. For example, the polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as P*cor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins (see, for example, Hill-Perkins and Possee, *J. Gen. Virol. 71*:971 (1990), Bonning, *et al., J. Gen. Virol. 75*:1551 (1994), and Chazenbalk and Rapoport, *J. Biol. Chem. 270*:1543 (1995). In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed that replace the native Zwnt5 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen Corporation; Carlsbad, CA), or baculovirus gp67 (PharMingen: San Diego, CA) can be used in constructs to replace the native Zwnt5 secretory signal sequence.

The recombinant virus or bacmid is used to transfect host cells. Suitable insect host cells include cell lines derived from IPLB-*Sf*-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as *Sf*9 (ATCC CRL 1711), *Sf*21AE, and *Sf*21 (Invitrogen Corporation; San Diego, CA), as well as *Drosophila* Schneider-2 cells, and the HIGH FIVEO cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent No. 5,300,435). Commercially available serum-free media can be used to grow and to maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cellO405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. When recombinant virus is used, the cells are typically grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3.

Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*, "The baculovirus expression system," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Fungal cells, including yeast cells, can also be used to express the genes described herein. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Suitable promoters for expression in yeast include promoters from *GAL1* (galactose), *PGK* (phosphoglycerate kinase), *ADH* (alcohol dehydrogenase), *AOX1* (alcohol oxidase), HIS4 (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311, Kawasaki *et al.*, U.S. Patent No. 4,931,373, Brake, U.S. Patent No. 4,870,008, Welch *et al.*, U.S. Patent No. 5,037,743, and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (*e.g.*, leucine). A preferred vector system for use in *Saccharomyces*

cerevisiae is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Additional suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311, Kingsman *et al.*, U.S. Patent No. 4,615,974, and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446, 5,063,154, 5,139,936, and 4,661,454.

Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al., J. Gen. Microbiol. 132*:3459 (1986), and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

For example, the use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed by Raymond, U.S. Patent No. 5,716,808, Raymond, U.S. Patent No. 5,736,383, Raymond *et al., Yeast 14*:11-23 (1998), and in international publication Nos. WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), and which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. *P. methanolica* cells can be

transformed by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Expression vectors can also be introduced into plant protoplasts, intact plant tissues, or isolated plant cells. Methods for introducing expression vectors into plant tissue include the direct infection or co-cultivation of plant tissue with *Agrobacterium tumefaciens*, microprojectile-mediated delivery, DNA injection, electroporation, and the like. See, for example, Horsch *et al., Science 227*:1229 (1985), Klein *et al., Biotechnology 10*:268 (1992), and Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick *et al.* (eds.), pages 67-88 (CRC Press, 1993).

Alternatively, Zwnt5 genes can be expressed in prokaryotic host cells. Suitable promoters that can be used to express Zwnt5 polypeptides in a prokaryotic host are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacSpr*, *phoA*, and *lacZ* promoters of *E. coli*, promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol. 1*:277 (1987), Watson *et al., Molecular Biology of the Gene, 4th Ed.* (Benjamin Cummins 1987), and by Ausubel *et al.* (1995).

Preferred prokaryotic hosts include *E. coli* and *Bacillus subtilus*. Suitable strains of *E. coli* include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF', DH5IMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (ed.), *Molecular Biology Labfax* (Academic Press 1991)). Suitable strains of *Bacillus subtilus* include BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in *DNA Cloning: A Practical Approach*, Glover (ed.) (IRL Press 1985)).

When expressing a Zwnt5 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered

saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995), Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, page 137 (Wiley-Liss, Inc. 1995), and Georgiou, "Expression of Proteins in Bacteria," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), page 101 [John Wiley & Sons, Inc. 1996)].

Standard methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are provided, for example, by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system are provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard techniques for recovering protein produced by a bacterial system is provided by, for example, Grisshammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995).

7. Isolation of Zwnt5 Polypeptides

It is preferred to purify the polypeptides of the present invention to at least about 80% purity, more preferably to at least about 90% purity, even more preferably to at least about 95% purity, or even greater than 95% purity with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. The polypeptides of the present invention may also be purified to a pharmaceutically pure state, which is greater than 99.9% pure. Preferably, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

Fractionation and/or conventional purification methods can be used to obtain preparations of Zwnt5 purified from natural sources (*e.g.*, coronary artery smooth muscle tissue, placental tissue, uterine tissue, or tracheal tissue), and recombinant Zwnt5 polypeptides and fusion Zwnt5 polypeptides purified from recombinant host cells. In general, ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties.

Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Selection of a particular method for polypeptide isolation and purification is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology 1988), and Doonan, *Protein Purification Protocols* (The Humana Press 1996).

Additional variations in Zwnt5 isolation and purification can be devised by those of skill in the art. For example, anti-Zwnt5 antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification. The use of monoclonal antibody columns to purify interferons from recombinant cells and from natural sources has been described, for example, by Staehelin *et al., J. Biol. Chem. 256*:9750 (1981), and by Adolf *et al., J. Biol. Chem. 265*:9290 (1990). Moreover, methods for binding ligands, such as Zwnt5, to receptor polypeptides bound to support media are well known in the art.

The polypeptides of the present invention can also be isolated by exploitation of particular properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate [Sulkowski, *Trends in Biochem. 3*:1 (1985)]. Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography [M. Deutscher, (ed.), *Meth. Enzymol. 182*:529 (1990)]. Zwnt5 polypeptides or fragments thereof may also be prepared through chemical synthesis, as described below. Zwnt5 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; PEGylated or non-PEGylated; and may or may not include an initial methionine amino acid residue.

8. Chemical Synthesis of Zwnt5 Polypeptides

Zwnt5 polypeptides of the present invention can also be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. The polypeptides are preferably prepared by solid phase peptide synthesis, for example as described by Merrifield, *J. Am. Chem. Soc. 85*:2149 (1963). The synthesis is carried out with amino acids that are protected at the alpha-amino terminus. Trifunctional amino acids with labile side-chains are also protected with suitable groups to prevent undesired chemical reactions from occurring during the assembly of the polypeptides. The alpha-amino protecting group is selectively removed to allow subsequent reaction to take place at the amino-terminus. The conditions for the removal of the alpha-amino protecting group do not remove the side-chain protecting groups.

See Stewart *et al.*, "Solid Phase Peptide Synthesis" (2nd Edition), (Pierce Chemical Co. 1984), Bayer and Rapp, *Chem. Pept. Prot. 3*:3 (1986), Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach* (IRL Press 1989), and by Lloyd-Williams *et al., Chemical Approaches to the Synthesis of Peptides and Proteins* (CRC Press, Inc. 1997), Kaiser *et al., Anal. Biochem. 34*:595 (1970). The coupling reactions can be performed automatically with commercially available instruments such as ABI model 430A, 431A and 433A peptide synthesizers.

The "native chemical ligation" approach to producing polypeptides is one variation of total chemical synthesis strategy (see, for example, Dawson *et al., Science 266*:776 (1994), Hackeng *et al., Proc. Nat'l Acad. Sci. USA 94*:7845 (1997), and Dawson, *Methods Enzymol. 287*: 34 (1997)). According to this method, an N-terminal

cysteine-containing peptide is chemically ligated to a peptide having a C-terminal thioester group to form a normal peptide bond at the ligation site.

The "expressed protein ligation" method is a semi-synthesis variation of the ligation approach (see, for example, Muir *et al, Proc. Nat'l Acad. Sci. USA 95*:6705 (1998); Severinov and Muir, *J. Biol. Chem. 273*:16205 (1998)). Here, synthetic peptides and protein cleavage fragments are linked to form the desired protein product. This method is particularly useful for the site-specific incorporation of unnatural amino acids (*e.g.*, amino acids comprising biophysical or biochemical probes) into proteins.

In an approach illustrated by Muir *et al, Proc. Nat'l Acad. Sci. USA 95*:6705 (1998), a gene or gene fragment is cloned into the PCYB2-IMPACT vector (New England Biolabs, Inc.; Beverly, MA) using the *Nde*I and *Sma*I restriction sites. As a result, the gene or gene fragment is expressed in frame fused with a chitin binding domain sequence, and a Pro-Gly is appended to the native C terminus of the protein of interest. The presence of a C-terminal glycine reduces the chance of side reactions, because the glycine residue accelerates native chemical ligation. Affinity chromatography with a chitin resin is used to purify the expressed fusion protein, and the chemical ligation step is initiated by incubating the resin-bound protein with thiophenol and synthetic peptide in buffer. This mixture produces the *in situ* generation of a highly reactive phenyl $^{\alpha}$thioester derivative of the protein that rapidly ligates with the synthetic peptide to produce the desired semi-synthetic protein.

9. Assays for Zwnt5, Its Analogs, and the Zwnt5 Receptor

As described above, the disclosed polypeptides can be used to construct Zwnt5 variants. A Zwnt5 variant will possess a Zwnt5 biological activity, as determined by the *in vitro* assays described below. A polypeptide produced by a Zwnt5 variant gene is considered to be a Zwnt5 agonist if the polypeptide exhibits a biological activity

On the other hand, a Zsig9ε variant gene product that lacks biological activity may be a Zwnt5ε antagonist. These biologically inactive Zwnt5 variants can be initially identified on the basis of hybridization analysis, sequence identity determination, or by the ability to specifically bind anti-Zwnt5 antibody. A Zwnt5 antagonist can be further characterized by its ability to inhibit the biological response induced by Zwnt5 or by a Zwnt5 agonist. This inhibitory effect may result, for example, from the competitive or non-competitive binding of the antagonist to the Zwnt5 receptor.

Zwnt5, its agonists and antagonists are valuable in both *in vivo* and *in vitro* uses. As an illustration, cytokines can be used as components of defined cell culture media, alone or in combination with other cytokines and hormones, to replace

serum that is commonly used in cell culture. Antagonists are also useful as research reagents for characterizing sites of interaction between Zwnt5 and its receptor. In a therapeutic setting, pharmaceutical compositions comprising Zwnt5 antagonists can be used to inhibit Zwnt5 activity.

One general class of Zwnt5 analogs are agonists or antagonists having an amino acid sequence that is a mutation of the amino acid sequences disclosed herein. Another general class of Zwnt5 analogs is provided by anti-idiotype antibodies, and fragments thereof, as described below. Moreover, recombinant antibodies comprising anti-idiotype variable domains can be used as analogs (see, for example, Monfardini *et al., Proc. Assoc. Am. Physicians 108*:420 (1996)). Since the variable domains of anti-idiotype Zwnt5ε antibodies mimic Zwnt5, these domains can provide either Zwnt5 agonist or antagonist activity.

A third approach to identifying Zwnt5 analogs is provided by the use of combinatorial libraries. Methods for constructing and screening phage display and other combinatorial libraries are provided, for example, by Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press 1996), Verdine, U.S. Patent No. 5,783,384, Kay, *et. al.*, U.S. Patent No. 5,747,334, and Kauffman *et al.*, U.S. Patent No. 5,723,323.

As a receptor ligand, the activity of Zwnt5 can be measured by a silicon-based biosensor microphysiometer which measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent cellular responses. An exemplary device is the CYTOSENSOR Microphysiometer manufactured by Molecular Devices Corp. (Sunnyvale, CA). A variety of cellular responses, such as cell proliferation, ion transport, energy production, inflammatory response, regulatory and receptor activation, and the like, can be measured by this method (see, for example, McConnell *et al., Science 257*:1906 (1992), Pitchford *et al., Meth. Enzymol. 228*:84 (1997), Arimilli *et al., J. Immunol. Meth. 212*:49 (1998), and Van Liefde *et al., Eur. J. Pharmacol. 346*:87 (1998)). Moreover, the microphysiometer can be used for assaying adherent or non-adherent eukaryotic or prokaryotic cells.

Since energy metabolism is coupled with the use of cellular ATP, any event that alters cellular ATP levels, such as receptor activation and the initiation of signal transduction, will cause a change in cellular acid section. An early event in interferon signal transduction is protein phosphorylation, which requires ATP. By measuring extracellular acidification changes in cell media over time, therefore, the microphysiometer directly measures cellular responses to various stimuli, including Zwnt5, its agonists, or antagonists. Preferably, the microphysiometer is used to measure responses of a Zwnt5 responsive eukaryotic cell, compared to a control eukaryotic cell that does not respond to Zwnt5 polypeptide. Zwnt5 responsive eukaryotic cells comprise

cells into which a receptor for Zwnt5 has been transfected to create a cell that is responsive to Zwnt5, or cells that are naturally responsive to Zwnt5. Suitable Zwnt5 responsive cells include HeLa cells, Daudi cells, L929 cells (ATCC No. CCL-1), human lung carcinoma cells (*e.g.*, A549 cells; ATCC No. CCL-185) cells, and normal human diploid fibroblasts cells (*e.g.*, FS-4 cells; Vilcek *et al., J. Exp. Med. 163*:632 (1986)). Zwnt5 modulated cellular responses are measured by a change (*e.g.*, an increase or decrease in extracellular acidification) in the response of cells exposed to Zwnt5, compared with control cells that have not been exposed to Zwnt5.

Accordingly, a microphysiometer can be used to identify cells, tissues, or cell lines which respond to an Zwnt5 stimulated pathway, and which express a functional Zwnt5 receptor. As an illustration, cells that express a functional Zwnt5 receptor can be identified by (a) providing test cells, (b) incubating a first portion of the test cells in the absence of Zwnt5, (c) incubating a second portion of the test cells in the presence of Zwnt5, and (d) detecting a change (*e.g.*, an increase or decrease in extracellular acidification rate, as measured by a microphysiometer) in a cellular response of the second portion of the test cells, as compared to the first portion of the test cells, wherein such a change in cellular response indicates that the test cells express a functional Zwnt5 receptor. An additional negative control may be included in which a portion of the test cells is incubated with Zwnt5 and an anti-Zwnt5 antibody to inhibit the binding of Zwnt5 with its cognate receptor.

The microphysiometer also provides one means to identify Zwnt5 agonists. For example, agonists of Zwnt5 can be identified by a method, comprising the steps of (a) providing cells responsive to Zwnt5, (b) incubating a first portion of the cells in the absence of a test compound, (c) incubating a second portion of the cells in the presence of a test compound, and (d) detecting a change, for example, an increase or diminution, in a cellular response of the second portion of the cells as compared to the first portion of the cells, wherein such a change in cellular response indicates that the test compound is an Zwnt5 agonist. An illustrative change in cellular response is a measurable change in extracellular acidification rate, as measured by a microphysiometer. Moreover, incubating a third portion of the cells in the presence of Zwnt5 and in the absence of a test compound can be used as a positive control for the Zwnt5 responsive cells, and as a control to compare the agonist activity of a test compound with that of Zwnt5. An additional control may be included in which a portion of the cells is incubated with a test compound (or Zwnt5) and an anti-Zwnt5 antibody to inhibit the binding of the test compound (or Zwnt5) with the Zwnt5 receptor.

The microphysiometer also provides a means to identify Zwnt5 antagonists. For example, Zwnt5 antagonists can be identified by a method, comprising

the steps of (a) providing cells responsive to Zwnt5, (b) incubating a first portion of the cells in the presence of Zwnt5 and in the absence of a test compound, (c) incubating a second portion of the cells in the presence of both Zwnt5 and the test compound, and (d) comparing the cellular responses of the first and second cell portions, wherein a decreased response by the second portion, compared with the response of the first portion, indicates that the test compound is an Zwnt5 antagonist. An illustrative change in cellular response is a measurable change extracellular acidification rate, as measured by a microphysiometer.

Zwnt5, its analogs, and anti-iodiotype Zwnt5 antibodies can be used to identify and to isolate Zwnt5 receptors. For example, proteins and peptides of the present invention can be immobilized on a column and used to bind receptor proteins from membrane preparations that are run over the column (Hermanson *et al.* (eds.), *Immobilized Affinity Ligand Techniques*, pages 195-202 (Academic Press 1992)). Radiolabeled or affinity labeled Zwnt5 polypeptides can also be used to identify or to localize Zwnt5 receptors in a biological sample (see, for example, Deutscher (ed.), *Methods in Enzymol.*, vol. 182, pages 721-37 (Academic Press 1990); Brunner *et al.*, *Ann. Rev. Biochem. 62*:483 (1993); Fedan *et al., Biochem. Pharmacol. 33*:1167 (1984)). Also see, Varthakavi and Minocha, *J. Gen. Virol. 77*:1875 (1996), who describe the use of anti-idiotype antibodies for receptor identification.

In addition, a solid phase system can be used to identify a Zwnt5 receptor, or an agonist or antagonist of a Zwnt5 receptor. For example, a Zwnt5 polypeptide or Zwnt5 fusion protein can be immobilized onto the surface of a receptor chip of a commercially available biosensor instrument (BIACORE, Biacore AB; Uppsala, Sweden). The use of this instrument is disclosed, for example, by Karlsson, *Immunol. Methods 145*:229 (1991), and Cunningham and Wells, *J. Mol. Biol. 234*:554 (1993).

As an illustration, a Zwnt5 polypeptide or fusion protein is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within a flow cell. A test sample is then passed through the cell. If a receptor is present in the sample, it will bind to the immobilized polypeptide or fusion protein, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding. This system can also be used to examine antibody-antigen interactions, and the interactions of other complement/anti-complement pairs.

10. Production of Antibodies to Zwnt5 Proteins

Antibodies to Zwnt5 can be obtained, for example, using the product of a Zwnt5 expression vector or Zwnt5 isolated from a natural source as an antigen. Particularly useful anti-Zwnt5 antibodies "bind specifically" with Zwnt5. Antibodies are considered to be specifically binding if the antibodies exhibit at least one of the following two properties: (1) antibodies bind to Zwnt5 with a threshold level of binding activity, and (2) antibodies do not significantly cross-react with polypeptides related to Zwnt5.

With regard to the first characteristic, antibodies specifically bind if they bind to a Zwnt5 polypeptide, peptide or epitope with a binding affinity (K_a) of $10^6\ M^{-1}$ or greater, preferably $10^7\ M^{-1}$ or greater, more preferably $10^8\ M^{-1}$ or greater, and most preferably $10^9\ M^{-1}$ or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, *Ann. NY Acad. Sci. 51*:660 (1949)). With regard to the second characteristic, antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zwnt5, but not known related polypeptides using a standard Western blot analysis. Examples of known related polypeptides are orthologs and proteins from the same species that are members of a protein family.

Anti-Zwnt5 antibodies can be produced using antigenic Zwnt5ε epitope-bearing peptides and polypeptides. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained within SEQ ID NO: 2. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that bind with Zwnt5. It is desirable that the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues, while hydrophobic residues are preferably avoided). Moreover, amino acid sequences containing proline residues may be also be desirable for antibody production.

As an illustration, potential antigenic sites in human Zwnt5 were identified using the Jameson-Wolf method, Jameson and Wolf, *CABIOS 4*:181, (1988), as implemented by the PROTEAN program (version 3.14) of LASERGENE (DNASTAR; Madison, WI). Default parameters were used in this analysis.

The Jameson-Wolf method predicts potential antigenic determinants by combining six major subroutines for protein structural prediction. Briefly, the Hopp-Woods method, Hopp *et al., Proc. Nat'l Acad. Sci. USA 78*:3824 (1981), was first used

to identify amino acid sequences representing areas of greatest local hydrophilicity (parameter: seven residues averaged). In the second step, Emini's method, Emini *et al.*, *J. Virology 55*:836 (1985), was used to calculate surface probabilities (parameter: surface decision threshold (0.6) = 1). Third, the Karplus-Schultz method, Karplus and Schultz, *Naturwissenschaften 72*:212 (1985), was used to predict backbone chain flexibility (parameter: flexibility threshold (0.2) = 1). In the fourth and fifth steps of the analysis, secondary structure predictions were applied to the data using the methods of Chou-Fasman, Chou, "Prediction of Protein Structural Classes from Amino Acid Composition," in *Prediction of Protein Structure and the Principles of Protein Conformation*, Fasman (ed.), pages 549-586 (Plenum Press 1990), and Garnier-Robson, Garnier *et al., J. Mol. Biol. 120*:97 (1978) (Chou-Fasman parameters: conformation table = 64 proteins; α region threshold = 103; β region threshold = 105; Garnier-Robson parameters: α and β decision constants = 0). In the sixth subroutine, flexibility parameters and hydropathy/solvent accessibility factors were combined to determine a surface contour value, designated as the "antigenic index." Finally, a peak broadening function was applied to the antigenic index, which broadens major surface peaks by adding 20, 40, 60, or 80% of the respective peak value to account for additional free energy derived from the mobility of surface regions relative to interior regions. This calculation was not applied, however, to any major peak that resides in a helical region, since helical regions tend to be less flexible.

The results of this analysis indicated that the following amino acid sequences of SEQ ID NO: 2 would provide suitable antigenic peptides: amino acids 34 to 77 of SEQ ID NO: 2 (SEQ ID NO: 4), amino acids 66 to 96 (SEQ ID NO: 5), 73 to 119 (SEQ ID NO: 6), amino acids 80 to 115 (SEQ ID NO: 7), amino acids 100 to 156 (SEQ ID NO: 8), amino acids 143 to 175 (SEQ ID NO: 9), amino acids 150 to 195 (SEQ ID NO: 10), amino acids 168 to 198 (SEQ ID NO: 11), amino acids 184 to 215 (SEQ ID NO: 12), amino acids 198 to 237 (SEQ ID NO: 13), amino acids 195 to 258 (SEQ ID NO: 14), amino acids 185 to 276 (SEQ ID NO: 15), amino acids 189 to 299 (SEQ ID NO: 16), amino acids 168 to 304 (SEQ ID NO: 17), amino acids 184 to 327 (SEQ ID NO: 18), amino acids 34 to 63 (SEQ ID NO: 19), and amino acids 73 to 102 (SEQ ID NO: 20). The present invention contemplates the use of any one of antigenic polypeptides to generate antibodies to Zwnt5.

Polyclonal antibodies to recombinant Zwnt5 protein or to Zwnt5 isolated from natural sources can be prepared using methods well-known to those of skill in the art. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992), and Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and

purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995). The immunogenicity of a Zwnt5 polypeptide can be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zwnt5 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like," such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

Although polyclonal antibodies are typically raised in animals such as horses, cows, dogs, chicken, rats, mice, rabbits, guinea pigs, goats, or sheep, an anti-Zwnt5 antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465, and in Losman *et al., Int. J. Cancer 46*:310 (1990).

Alternatively, monoclonal anti-Zwnt5 antibodies can be generated. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art [see, for example, Kohler *et al., Nature 256*:495 (1975), Coligan *et al.* (eds.), *Current Protocols in Immunology, Vol. 1*, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"], Picksley *et al.*, "Production of monoclonal antibodies against proteins expressed in *E. coli*," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 93 (Oxford University Press 1995)].

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an *Zwnt5* gene product, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-Zwnt5 antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The

transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green *et al., Nature Genet. 7*:13 (1994), Lonberg *et al., Nature 368*:856 (1994), and Taylor *et al., Int. Immun. 6*:579 (1994).

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography [see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in *Methods in Molecular Biology, Vol. 10*, pages 79-104 (The Humana Press, Inc. 1992)].

For particular uses, it may be desirable to prepare fragments of anti-Zwnt5 antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff *et al., Arch Biochem. Biophys. 89*:230 (1960), Porter, *Biochem. J. 73*:119 (1959), Edelman *et al.*, in *Methods in Enzymology Vol. 1*, page 422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described by Inbar *et al., Proc. Nat'l Acad. Sci. USA 69*:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (see, for example, Sandhu, *Crit. Rev. Biotech. 12*:437 (1992)).

The Fv fragments may comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L

domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are described, for example, by Whitlow *et al., Methods: A Companion to Methods in Enzymology* 2:97 (1991) (also see, Bird *et al., Science 242*:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al., Bio/Technology 11*:1271 (1993), and Sandhu, *supra*).

As an illustration, a scFV can be obtained by exposing lymphocytes to Zwnt5ε polypeptide *in vitro*, and selecting antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zwnt5 protein or peptide). Genes encoding polypeptides having potential Zwnt5 polypeptide-binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides that interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art [(Ladner *et al.*, U.S. Patent No. 5,223,409, Ladner *et al.*, U.S. Patent No. 4,946,778, Ladner *et al.*, U.S. Patent No. 5,403,484, Ladner *et al.*, U.S. Patent No. 5,571,698, and Kay *et al., Phage Display of Peptides and Proteins* (Academic Press, Inc. 1996)] and random peptide display libraries and kits for screening such libraries are available commercially, for instance from CLONTECH Laboratories, Inc. (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA), and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zwnt5 sequences disclosed herein to identify proteins that bind to Zwnt5.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells [see, for example, Larrick *et al., Methods: A Companion to Methods in Enzymology* 2:106 (1991), Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter *et al.* (eds.), page 166 (Cambridge University Press 1995), and Ward *et al.*, "Genetic Manipulation and

Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch *et al.*, (eds.), page 137 (Wiley-Liss, Inc. 1995)].

Alternatively, an anti-Zwnt5 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain. Typical residues of human antibodies are then substituted in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al., Proc. Nat'l Acad. Sci. USA 86*:3833 (1989). Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al., Nature 321*:522 (1986), Carter *et al., Proc. Nat'l Acad. Sci. USA 89*:4285 (1992), Sandhu, *Crit. Rev. Biotech. 12*:437 (1992), Singer *et al.*, *J. Immun. 150*:2844 (1993), Sudhir (ed.), *Antibody Engineering Protocols* (Humana Press, Inc. 1995), Kelley, "Engineering Therapeutic Antibodies," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 399-434 (John Wiley & Sons, Inc. 1996), and by Queen *et al.*, U.S. Patent No. 5,693,762 (1997).

Polyclonal anti-idiotype antibodies can be prepared by immunizing animals with anti-Zwnt5 antibodies or antibody fragments, using standard techniques. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Methods In Molecular Biology: Immunochemical Protocols*, Manson (ed.), pages 1-12 (Humana Press 1992). Also, see Coligan at pages 2.4.1-2.4.7. Alternatively, monoclonal anti-idiotype antibodies can be prepared using anti-Zwnt5 antibodies or antibody fragments as immunogens with the techniques, described above. As another alternative, humanized anti-idiotype antibodies or subhuman primate anti-idiotype antibodies can be prepared using the above-described techniques. Methods for producing anti-idiotype antibodies are described, for example, by Irie, U.S. Patent No. 5,208,146, Greene, *et. al.*, U.S. Patent No. 5,637,677, and Varthakavi and Minocha, *J. Gen. Virol. 77*:1875 (1996).

11. Diagnostic Application of Zwnt5 Nucleotide Sequences

Nucleic acid molecules can be used to detect the expression of an *Zwnt5* gene in a biological sample. Although such probe molecules can include murine Zwnt5 encoding sequences, preferred probe molecules include double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the

nucleotide sequence of SEQ ID NO: 1 or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like.

In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target Zwnt5 RNA species. After separating unbound probe from hybridized molecules, the amount of hybrids is detected. Illustrative biological samples include blood, urine, saliva, tissue biopsy, and autopsy material.

Well-established hybridization methods of RNA detection include northern analysis and dot/slot blot hybridization (see, for example, Ausubel (1995) at pages 4-1 to 4-27, and Wu *et al.* (eds.), "Analysis of Gene Expression at the RNA Level," in *Methods in Gene Biotechnology*, pages 225-239 (CRC Press, Inc. 1997)). Nucleic acid probes can be detectably labeled with radioisotopes such as ^{32}P or ^{35}S. Alternatively, *Zwnt5* RNA can be detected with a nonradioactive hybridization method (see, for example, Isaac (ed.), *Protocols for Nucleic Acid Analysis by Nonradioactive Probes* (Humana Press, Inc. 1993)). Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative nonradioactive moieties include biotin, fluorescein, and digoxigenin.

*Zwnt5*oligonucleotide probes are also useful for *in vivo* diagnosis. As an illustration, ^{18}F-labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography [Tavitian *et al., Nature Medicine 4*:467 (1998)].

Numerous diagnostic procedures take advantage of the polymerase chain reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known [see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.), *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 1998)].

One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample, reverse transcribed to cDNA, and the cDNA is incubated with Zwnt5 primers [see, for example, Wu *et al.* (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in *Methods in Gene Biotechnology*, pages 15-28 (CRC Press, Inc. 1997)]. PCR is then performed and the products are analyzed using standard techniques.

As an illustration, RNA is isolated from biological sample using, for example, the gunadinium-thiocyanate cell lysis procedure described above.

Alternatively, a solid-phase technique can be used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random oligonucleotides, short homopolymers of dT, or *Zwnt5*anti-sense oligomers. Oligo-dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. Zwnt5 sequences are amplified by the polymerase chain reaction using two flanking oligonucleotide primers that are typically 20 bases in length.

PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can be transferred to a membrane, hybridized with a detectably-labeled Zwnt5 probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay.

Another approach for detection of Zwnt5 expression is cycling probe technology (CPT), in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNAase H, and the presence of cleaved chimeric probe is detected [see, for example, Beggs *et al.*, *J. Clin. Microbiol. 34*:2985 (1996), Bekkaoui *et al.*, *Biotechniques 20*:240 (1996)]. Alternative methods for detection of Zwnt5 sequences can utilize approaches such as nucleic acid sequence-based amplification (NASBA), cooperative amplification of templates by cross-hybridization (CATCH), and the ligase chain reaction (LCR) (see, for example, Marshall *et al.*, U.S. Patent No. 5,686,272 (1997), Dyer *et al.*, *J. Virol. Methods 60*:161 (1996), Ehricht *et al.*, *Eur. J. Biochem. 243*:358 (1997), and Chadwick *et al.*, *J. Virol. Methods 70*:59 (1998)). Other standard methods are known to those of skill in the art.

Zwnt5 probes and primers can also be used to detect and to localize *Zwnt5* gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), *In Situ Hybridization Protocols* (Humana Press, Inc. 1994), Wu *et al.* (eds.), "Analysis of Cellular DNA or Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 259-278 (CRC Press, Inc. 1997), and Wu *et al.* (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 279-289 (CRC Press, Inc. 1997)). Various additional diagnostic approaches are well-known to those of skill in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc.

1991), Coleman and Tsongalis, *Molecular Diagnostics* (Humana Press, Inc. 1996), and Elles, *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc., 1996)).

Nucleic acid molecules comprising *Zwnt5* nucleotide sequences can also be used to determine whether a subject's chromosomes contain a mutation in the *Zwnt5* gene. Detectable chromosomal aberrations at the *Zwnt5* gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Of particular interest are genetic alterations that inactivate the *Zwnt5* gene.

Aberrations associated with the *Zwnt5* locus can be detected using nucleic acid molecules of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, amplification-refractory mutation system analysis (ARMS), single-strand conformation polymorphism (SSCP) detection, RNase cleavage methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis (FAMA), and other genetic analysis techniques known in the art [see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest 108*:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Human Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), *Genome Analysis, Vol. 2: Detecting Genes* (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and Ward, "Molecular Diagnostic Testing," in *Principles of Molecular Medicine*, pages 83-88 (Humana Press, Inc. 1998)].

The protein truncation test is also useful for detecting the inactivation of a gene in which translation-terminating mutations produce only portions of the encoded protein [see, for example, Stoppa-Lyonnet *et al., Blood 91*:3920 (1998)]. According to this approach, RNA is isolated from a biological sample, and used to synthesize cDNA. PCR is then used to amplify the *Zwnt5* target sequence and to introduce an RNA polymerase promoter, a translation initiation sequence, and an in-frame ATG triplet. PCR products are transcribed using an RNA polymerase, and the transcripts are translated *in vitro* with a T7-coupled reticulocyte lysate system. The translation products are then fractionated by SDS-PAGE to determine the lengths of the translation products. The protein truncation test is described, for example, by Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics,* pages 9.11.1 - 9.11.18 (John Wiley & Sons 1998).

In a related approach, Zwnt5 protein is isolated from a subject, the molecular weight of the isolated Zwnt5 protein is determined, and then compared with

the molecular weight a normal Zwnt5 protein, such as a protein having the amino acid sequence of SEQ ID NO:2. A substantially lower molecular weight for the isolated Zwnt5 protein is indicative that the protein is truncated. In this context, "substantially lower molecular weight" refers to at least about 10 percent lower, and preferably, at least about 25 percent lower. The Zwnt5 protein may be isolated by various procedures known in the art including immunoprecipitation, solid phase radioimmunoassay, enzyme-linked immunosorbent assay, or Western blotting. The molecular weight of the isolated Zwnt5 protein can be determined using standard techniques, such as SDS-polyacrylamide gel electrophoresis.

The present invention also contemplates kits for performing a diagnostic assay for *Zwnt5* gene expression or to detect mutations in the *Zwnt5ε* gene. Such kits comprise nucleic acid probes, such as double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like. Kits may comprise nucleic acid primers for performing PCR.

Preferably, such a kit contains all the necessary elements to perform a nucleic acid diagnostic assay described above. A kit will comprise at least one container comprising a *Zwnt5* probe or primer. The kit may also comprise a second container comprising one or more reagents capable of indicating the presence of *Zwnt5* sequences. Examples of such indicator reagents include detectable labels such as radioactive labels, fluorochromes, chemiluminescent agents, and the like. A kit may also comprise a means for conveying to the user that the Zwnt5 probes and primers are used to detect *Zwnt5* gene expression. For example, written instructions may state that the enclosed nucleic acid molecules can be used to detect either a nucleic acid molecule that encodes Zwnt5, or a nucleic acid molecule having a nucleotide sequence that is complementary to a Zwnt5-encoding nucleotide sequence. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

12. Diagnostic Application of Anti-Zwnt5 Antibodies

The present invention contemplates the use of anti-Zwnt5 antibodies to screen biological samples *in vitro* for the presence of Zwnt5. In one type of *in vitro* assay, anti-Zwnt5 antibodies are used in liquid phase. For example, the presence of Zwnt5 in a biological sample can be tested by mixing the biological sample with a trace amount of labeled Zwnt5 and an anti-Zwnt5 antibody under conditions that promote binding between

Zwnt5 and its antibody. Complexes of Zwnt5 and anti-Zwnt5 in the sample can be separated from the reaction mixture by contacting the complex with an immobilized protein which binds with the antibody, such as an Fc antibody or *Staphylococcus* protein A. The concentration of Zwnt5 in the biological sample will be inversely proportional to the amount of labeled Zwnt5 bound to the antibody and directly related to the amount of free-labeled Zwnt5. Illustrative biological samples include blood, urine, saliva, tissue biopsy, and autopsy material.

Alternatively, *in vitro* assays can be performed in which anti-Zwnt5 antibody is bound to a solid-phase carrier. For example, antibody can be attached to a polymer, such as aminodextran, in order to link the antibody to an insoluble support such as a polymer-coated bead, a plate or a tube. Other suitable *in vitro* assays will be readily apparent to those of skill in the art.

In another approach, anti-Zwnt5 antibodies can be used to detect Zwnt5ε in tissue sections prepared from a biopsy specimen. Such immunochemical detection can be used to determine the relative abundance of Zwnt5 and to determine the distribution of Zwnt5 in the examined tissue. General immunochemistry techniques are well established [see, for example, Ponder, "Cell Marking Techniques and Their Application," in *Mammalian Development: A Practical Approach*, Monk (ed.), pages 115-38 (IRL Press 1987), Coligan at pages 5.8.1-5.8.8, Ausubel (1995) at pages 14.6.1 to 14.6.13 (Wiley Interscience 1990), and Manson (ed.), *Methods In Molecular Biology, Vol. 10: Immunochemical Protocols* (The Humana Press, Inc. 1992)].

Immunochemical detection can be performed by contacting a biological sample with an anti-Zwnt5 antibody, and then contacting the biological sample with a detectably labeled molecule that binds to the antibody. For example, the detectably labeled molecule can comprise an antibody moiety that binds to anti-Zwnt5 antibody. Alternatively, the anti-Zwnt5 antibody can be conjugated with avidin/streptavidin (or biotin) and the detectably labeled molecule can comprise biotin (or avidin/streptavidin).

Alternatively, an anti-Zwnt5 antibody can be conjugated with a detectable label to form an anti-Zwnt5 immunoconjugate. Suitable detectable labels include, for example, a radioisotope, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label or colloidal gold. Methods of making and detecting such detectably labeled immunoconjugates are described in more detail below.

The detectable label can be a radioisotope that is detected by autoradiography. Isotopes that are particularly useful for the purpose of the present invention are ^{3}H, ^{125}I, ^{131}I, ^{35}S and ^{14}C.

Anti-Zwnt5 immunoconjugates can also be labeled with a fluorescent compound. The presence of a fluorescently labeled antibody is determined by exposing the

immunoconjugate to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, anti-Zwnt5 immunoconjugates can be detectably labeled by coupling an antibody component to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoconjugate is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

Similarly, a bioluminescent compound can be used to label anti-Zwnt5 immunoconjugates of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

Alternatively, anti-Zwnt5 immunoconjugates can be detectably labeled by linking an anti-Zwnt5 antibody component to an enzyme. When the anti-Zwnt5-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label polyspecific immunoconjugates include β-galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

The binding of marker moieties to anti-Zwnt5 antibodies can be accomplished using standard techniques. Typical methodology in this regard is described by Kennedy *et al., Clin. Chim. Acta 70*:1 (1976), Schurs *et al., Clin. Chim. Acta 81*:1 (1977), Shih *et al., Int'l J. Cancer 46*:1101 (1990), Stein *et al., Cancer Res. 50*:1330 (1990), and Coligan, *supra.*

Moreover, the convenience and versatility of immunochemical detection can be enhanced by using anti-Zwnt5 antibodies that have been conjugated with avidin, streptavidin, and biotin (see, for example, Wilchek *et al.* (eds.), "Avidin-Biotin Technology," *Methods In Enzymology, Vol. 184* (Academic Press 1990), and Bayer *et al.*, "Immunochemical Applications of Avidin-Biotin Technology," in *Methods In Molecular Biology, Vol. 10*, Manson (ed.), pages 149-162 (The Humana Press, Inc. 1992).

Methods for performing immunoassays are illustrated in the following: Cook and Self, "Monoclonal Antibodies in Diagnostic Immunoassays," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 180-208, (Cambridge University Press, 1995), Perry, "The Role of Monoclonal

Antibodies in the Advancement of Immunoassay Technology," in *Monoclonal Antibodies: Principles and Applications*, Birch and Lennox (eds.), pages 107-120 (Wiley-Liss, Inc. 1995), and Diamandis, *Immunoassay* (Academic Press, Inc. 1996).

In a related approach, biotin- or FITC-labeled Zwnt5 can be used to identify cells that bind Zwnt5. Such can binding can be detected, for example, using flow cytometry.

The present invention also contemplates kits for performing an immunological diagnostic assay for Zwnt5 gene expression. Such kits comprise at least one container comprising an anti-Zwnt5 antibody, or antibody fragment. A kit may also comprise a second container comprising one or more reagents capable of indicating the presence of Zwnt5 antibody or antibody fragments. Examples of such indicator reagents include detectable labels such as a radioactive label, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label, colloidal gold, and the like. A kit may also comprise a means for conveying to the user that Zwnt5 antibodies or antibody fragments are used to detect Zwnt5 protein. For example, written instructions may state that the enclosed antibody or antibody fragment can be used to detect Zwnt5. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

13. Therapeutic Uses of Polypeptides Having Zwnt5 Activity

Generally, the dosage of administered Zwnt5 (or Zwnt5 analog or fusion protein) will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of Zwnt5 that is in the range of from about 1 pg/kg to 10 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage also may be administered as circumstances dictate.

Administration of a molecule having Zwnt5 activity to a subject can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering therapeutic proteins by injection, the administration may be by continuous infusion or by single or multiple boluses. Alternatively, Zwnt5 can be administered as a controlled release formulation. Additional routes of administration include oral, dermal, mucosal-membrane, pulmonary, and transcutaneous. Oral delivery is suitable for polyester microspheres, zein microspheres, proteinoid microspheres, polycyanoacrylate microspheres, and lipid-based systems [see, for example, DiBase and

Morrel, "Oral Delivery of Microencapsulated Proteins," in *Protein Delivery: Physical Systems*, Sanders and Hendren (eds.), pages 255-288 (Plenum Press 1997)]. The feasibility of an intranasal delivery is exemplified by such a mode of insulin administration [see, for example, Hinchcliffe and Illum, *Adv. Drug Deliv. Rev. 35*:199 (1999)]. Dry or liquid particles comprising Zwnt5ε can be prepared and inhaled with the aid of dry-powder dispersers, liquid aerosol generators, or nebulizers [*e.g.*, Pettit and Gombotz, *TIBTECH 16*:343 (1998); Patton *et al., Adv. Drug Deliv. Rev. 35*:235 (1999)]. This approach is illustrated by the AERX diabetes management system, which is a hand-held electronic inhaler that delivers aerosolized insulin into the lungs. Studies have shown that proteins as large as 48,000 kDa have been delivered across skin at therapeutic concentrations with the aid of low-frequency ultrasound, which illustrates the feasibility of trascutaneous administration [Mitragotri *et al., Science 269*:850 (1995)]. Transdermal delivery using electroporation provides another means to administer Zwnt5 [Potts *et al., Pharm. Biotechnol. 10*:213 (1997)].

A pharmaceutical composition comprising a protein, polypeptide, or peptide having Zwnt5 activity can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the therapeutic proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. See, for example, Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995).

For purposes of therapy, molecules having Zwnt5 activity and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A combination of a protein, polypeptide, or peptide having Zwnt5 activity and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. A pharmaceutical composition comprising molecules having Zwnt5activity can be furnished in liquid form, in an aerosol, or in solid form. Proteins having Zwnt5 activity, such as human or murine Zwnt5ε, can be administered as a conjugate with a pharmaceutically acceptable water-soluble polymer moiety, as described above. Liquid forms, including liposome-encapsulated formulations, are illustrated by injectable solutions and oral suspensions. Exemplary solid forms include capsules, tablets, and controlled-release forms, such as a miniosmotic pump or an implant. Other dosage forms can be devised by those skilled in

the art, as shown, for example, by Ansel and Popovich, *Pharmaceutical Dosage Forms and Drug Delivery Systems,* 5th Edition (Lea & Febiger 1990), Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995), and by Ranade and Hollinger, *Drug Delivery Systems* (CRC Press 1996).

As an illustration, Zwnt5 pharmaceutical compositions may be supplied as a kit comprising a container that comprises Zwnt5, a Zwnt5 agonist, or a Zwnt5 antagonist (*e.g.,* an anti-Zwnt5 antibody or antibody fragment). Zwnt5 can be provided in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Alternatively, such a kit can include a dry-powder disperser, liquid aerosol generator, or nebulizer for administration of a therapeutic polypeptide. Such a kit may further comprise written information on indications and usage of the pharmaceutical composition. Moreover, such information may include a statement that the Zwnt5 composition is contraindicated in patients with known hypersensitivity to Zwnt5.

14. Therapeutic Uses of Zwnt5 Nucleotide Sequences

Immunomodulator genes can be introduced into a subject to enhance immunological responses. As an illustration "immunomodulator gene therapy" has been examined in model systems using vectors that express IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-15, tumor necrosis factor-α, or granulocyte-macrophage colony-stimulating factor (see, for example, Cao *et al., J. Gastroenterol. Hepatol. 11*:1053 (1996), Tahara *et al., Ann. N. Y. Acad. Sci. 795*:275 (1996), Rakhmilevich *et al., Hum. Gene Ther. 8*:1303 (1997), and Cao *et al., Transplantation 65*:325 (1998)). In addition, a therapeutic expression vector can be provided that inhibits *Zwnt5* gene expression, such as an anti-sense molecule, a ribozyme, or an external guide sequence molecule.

There are numerous approaches to introduce an *Zwnt5* gene to a subject, including the use of recombinant host cells that express Zwnt5, delivery of naked nucleic acid encoding Zwnt5, use of a cationic lipid carrier with a nucleic acid molecule that encodes Zwnt5, and the use of viruses that express Zwnt5, such as recombinant retroviruses, recombinant adeno-associated viruses, recombinant adenoviruses, and recombinant Herpes simplex viruses [HSV] [see, for example, Mulligan, *Science 260*:926 (1993), Rosenberg *et al., Science 242*:1575 (1988), LaSalle *et al., Science 259*:988 (1993), Wolff *et al., Science 247*:1465 (1990), Breakfield and Deluca, *The New Biologist 3*:203 (1991)]. In an *ex vivo* approach, for example, cells are isolated from a subject, transfected with a vector that expresses a Zwnt5 gene, and then transplanted into the subject.

In order to effect expression of a *Zwnt5* gene, an expression vector is constructed in which a nucleotide sequence encoding a *Zwnt5* gene is operably linked to a core promoter, and optionally a regulatory element, to control gene transcription. The general requirements of an expression vector are described above.

Alternatively, a *Zwnt5*gene can be delivered using recombinant viral vectors, including for example, adenoviral vectors [*e.g.*, Kass-Eisler *et al., Proc. Nat'l Acad. Sci. USA 90*:11498 (1993), Kolls *et al., Proc. Nat'l Acad. Sci. USA 91*:215 (1994), Li *et al., Hum. Gene Ther. 4*:403 (1993), Vincent *et al., Nat. Genet. 5*:130 (1993), and Zabner *et al., Cell 75*:207 (1993)], adenovirus-associated viral vectors (Flotte *et al., Proc. Nat'l Acad. Sci. USA 90*:10613 (1993)], alphaviruses such as Semliki Forest Virus and Sindbis Virus [Hertz and Huang, *J. Vir. 66*:857 (1992), Raju and Huang, *J. Vir. 65*:2501 (1991), and Xiong *et al., Science* 243:1188 (1989)], herpes viral vectors [*e.g.*, U.S. Patent Nos. 4,769,331, 4,859,587, 5,288,641 and 5,328,688), parvovirus vectors (Koering *et al., Hum. Gene Therap. 5*:457 (1994)], pox virus vectors [Ozaki *et al., Biochem. Biophys. Res. Comm. 193*:653 (1993), Panicali and Paoletti, *Proc. Nat'l Acad. Sci. USA 79*:4927 (1982)], pox viruses, such as canary pox virus or vaccinia virus [Fisher-Hoch *et al., Proc. Nat'l Acad. Sci. USA 86*:317 (1989), and Flexner *et al., Ann. N.Y. Acad. Sci. 569*:86 (1989)], and retroviruses [*e.g.*, Baba *et al., J. Neurosurg 79*:729 (1993), Ram *et al., Cancer Res. 53*:83 (1993), Takamiya *et al., J. Neurosci. Res 33*:493 (1992), Vile and Hart, *Cancer Res. 53*:962 (1993), Vile and Hart, *Cancer Res. 53*:3860 (1993), and Anderson *et al.,* U.S. Patent No. 5,399,346]. Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

As an illustration of one system, adenovirus, a double-stranded DNA virus, is a well-characterized gene transfer vector for delivery of a heterologous nucleic acid molecule [for a review, see Becker *et al., Meth. Cell Biol. 43*:161 (1994); Douglas and Curiel, *Science & Medicine 4*:44 (1997)]. The adenovirus system offers several advantages including: (i) the ability to accommodate relatively large DNA inserts, (ii) the ability to be grown to high-titer, (iii) the ability to infect a broad range of mammalian cell types, and (iv) the ability to be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. In addition, adenoviruses can be administered by intravenous injection, because the viruses are stable in the bloodstream.

Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell. When intravenously administered to intact animals,

adenovirus primarily targets the liver. Although an adenoviral delivery system with an E1 gene deletion cannot replicate in the host cells, the host's tissue will express and process an encoded heterologous protein. Host cells will also secrete the heterologous protein if the corresponding gene includes a secretory signal sequence. Secreted proteins will enter the circulation from tissue that expresses the heterologous gene (*e.g.*, the highly vascularized liver).

Moreover, adenoviral vectors containing various deletions of viral genes can be used to reduce or eliminate immune responses to the vector. Such adenoviruses are E1-deleted, and in addition, contain deletions of E2A or E4 [Lusky *et al., J. Virol. 72*:2022 (1998); Raper *et al., Human Gene Therapy 9*:671 (1998)]. The deletion of E2b has also been reported to reduce immune responses [Amalfitano *et al., J. Virol. 72*:926 (1998)]. By deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses, where all viral genes are deleted, are particularly advantageous for insertion of large inserts of heterologous DNA [for a review, see Yeh. and Perricaudet, *FASEB J. 11*:615 (1997)].

High titer stocks of recombinant viruses capable of expressing a therapeutic gene can be obtained from infected mammalian cells using standard methods. For example, recombinant HSV can be prepared in Vero cells, as described by Brandt *et al., J. Gen. Virol. 72*:2043 (1991), Herold *et al., J. Gen. Virol. 75*:1211 (1994), Visalli and Brandt, *Virology 185*:419 (1991), Grau *et al., Invest. Ophthalmol. Vis. Sci. 30*:2474 (1989), Brandt *et al., J. Virol. Meth. 36*:209 (1992), and by Brown and MacLean (eds.), *HSV Virus Protocols* (Humana Press 1997).

Alternatively, an expression vector comprising a *Zwnt5* gene can be introduced into a subject's cells by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner *et al., Proc. Nat'l Acad. Sci. USA 84*:7413 (1987); Mackey *et al., Proc. Nat'l Acad. Sci. USA 85*:8027 (1988)]. The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Liposomes can be used to direct transfection to particular cell types, which is particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

Electroporation is another alternative mode of administration. For example, Aihara and Miyazaki, *Nature Biotechnology 16*:867 (1998), have demonstrated the use of *in vivo* electroporation for gene transfer into muscle.

In an alternative approach to gene therapy, a therapeutic gene may encode a Zwnt5 anti-sense RNA that inhibits the expression of Zwnt5. Suitable sequences for anti-sense molecules can be derived from the nucleotide sequences of Zwnt5ε disclosed herein.

Alternatively, an expression vector can be constructed in which a regulatory element is operably linked to a nucleotide sequence that encodes a ribozyme. Ribozymes can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule (see, for example, Draper and Macejak, U.S. Patent No. 5,496,698, McSwiggen, U.S. Patent No. 5,525,468, Chowrira and McSwiggen, U.S. Patent No. 5,631,359, and Robertson and Goldberg, U.S. Patent No. 5,225,337). In the context of the present invention, ribozymes include nucleotide sequences that bind with Zwnt5 mRNA.

In another approach, expression vectors can be constructed in which a regulatory element directs the production of RNA transcripts capable of promoting RNase P-mediated cleavage of mRNA molecules that encode an *Zwnt5* gene. According to this approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, which is subsequently cleaved by the cellular ribozyme (see, for example, Altman *et al.,* U.S. Patent No. 5,168,053, Yuan *et al., Science 263*:1269 (1994), Pace *et al.,* international publication No. WO 96/18733, George *et al.,* international publication No. WO 96/21731, and Werner *et al.,* international publication No. WO 97/33991). Preferably, the external guide sequence comprises a ten to fifteen nucleotide sequence complementary to *Zwnt5* mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. The external guide sequence transcripts bind to the targeted mRNA species by the formation of base pairs between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the base-paired region.

In general, the dosage of a composition comprising a therapeutic vector having a *Zwnt5* nucleotide acid sequence, such as a recombinant virus, will vary depending upon such factors as the subject's age, weight, height, sex, general medical condition and previous medical history. Suitable routes of administration of therapeutic vectors include intravenous injection, intraarterial injection, intraperitoneal injection, and intramuscular injection.

A composition comprising viral vectors, non-viral vectors, or a combination of viral and non-viral vectors of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby vectors or viruses are combined in a mixture with a pharmaceutically acceptable carrier.

As noted above, a composition such as phosphate-buffered saline is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient subject. Other suitable carriers are well-known to those in the art [see, for example, *Remington's Pharmaceutical Sciences, 19th Ed.* (Mack Publishing Co. 1995), and *Gilman's the Pharmacological Basis of Therapeutics, 7th Ed.* (MacMillan Publishing Co. 1985)].

For purposes of therapy, a therapeutic gene expression vector, or a recombinant virus comprising such a vector, and a pharmaceutically acceptable carrier are administered to a subject in a therapeutically effective amount. A combination of an expression vector (or virus) and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject

When the subject treated with a therapeutic gene expression vector or a recombinant virus is a human, then the therapy is preferably somatic cell gene therapy. That is, the preferred treatment of a human with a therapeutic gene expression vector or a recombinant virus does not entail introducing into cells a nucleic acid molecule that can form part of a human germ line and be passed onto successive generations (*i.e.,* human germ line gene therapy).

15. Production of Transgenic Mice

Transgenic mice can be engineered to over-express the human or murine *Zwnt5*gene in all tissues or under the control of a tissue-specific or tissue-preferred regulatory element. These over-producers of Zwnt5 can be used to characterize the phenotype that results from over-expression, and the transgenic animals can serve as models for human disease caused by excess Zwnt5. Transgenic mice that over-express Zwnt5 also provide model bioreactors for production of Zwnt5 in the milk or blood of larger animals. Methods for producing transgenic mice are well-known to those of skill in the art [see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), Monastersky and Robl (eds.), *Strategies in Transgenic Animal Science* (ASM Press 1995), and Abbud and Nilson, "Recombinant Protein Expression in Transgenic Mice," in *Gene Expression Systems: Using Nature for the Art of Expression*, Fernandez and Hoeffler (eds.), pages 367-397 (Academic Press, Inc. 1999)].

For example, a method for producing a transgenic mouse that expresses a *Zwnt5* gene can begin with adult, fertile males (studs) [B6C3f1, 2-8 months of age (Taconic Farms, Germantown, NY)], vasectomized males (duds) [B6D2f1, 2-8 months, (Taconic Farms)], prepubescent fertile females (donors) [B6C3f1, 4-5 weeks, (Taconic Farms)] and adult fertile females (recipients) [B6D2f1, 2-4 months, (Taconic Farms)]. The donors are acclimated for one week and then injected with approximately 8 IU/mouse of Pregnant Mare's Serum gonadotrophin (Sigma Chemical Company; St. Louis, MO) I.P., and 46-47 hours later, 8 IU/mouse of human Chorionic Gonadotropin (hCG (Sigma)) I.P. to induce superovulation. Donors are mated with studs subsequent to hormone injections. Ovulation generally occurs within 13 hours of hCG injection. Copulation is confirmed by the presence of a vaginal plug the morning following mating.

Fertilized eggs are collected under a surgical scope. The oviducts are collected and eggs are released into urinanalysis slides containing hyaluronidase (Sigma). Eggs are washed once in hyaluronidase, and twice in Whitten's W640 medium [described, for example, by Menino and O'Claray, *Biol. Reprod. 77*:159 (1986), and Dienhart and Downs, *Zygote 4*:129 (1996)] that has been incubated with 5% CO_2, 5% O_2, and 90% N_2 at 37°C. The eggs are then stored in a 37°C/5% CO_2 incubator until microinjection.

Ten to twenty micrograms of plasmid DNA containing a Zwnt5-encoding sequence is linearized, gel-purified, and resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 mM EDTA (pH 8.0), at a final concentration of 5-10 nanograms per microliter for microinjection. Plasmid DNA is microinjected into harvested eggs contained in a drop of W640 medium overlaid by warm, CO_2-equilibrated mineral oil. The DNA is drawn into an injection needle (pulled from a 0.75mm ID, 1mm OD borosilicate glass capillary), and injected into individual eggs. Each egg is penetrated with the injection needle, into one or both of the haploid pronuclei. Picoliters of DNA are injected into the pronuclei, and the injection needle withdrawn without coming into contact with the nucleoli. The procedure is repeated until all the eggs are injected. Successfully microinjected eggs are transferred into an organ tissue-culture dish with pre-gassed W640 medium for storage overnight in a 37°C/5% CO_2 incubator.

The following day, two-cell embryos are transferred into pseudopregnant recipients. The recipients are identified by the presence of copulation plugs, after copulating with vasectomized duds. Recipients are anesthetized and shaved on the dorsal left side and transferred to a surgical microscope. A small incision is made in the skin and through the muscle wall in the middle of the abdominal area outlined by the ribcage, the saddle, and the hind leg, midway between knee and spleen. The reproductive organs are exteriorized onto a small surgical drape. The fat pad is stretched out over the

surgical drape, and a baby serrefine (Roboz, Rockville, MD) is attached to the fat pad and left hanging over the back of the mouse, preventing the organs from sliding back in.

With a fine transfer pipette containing mineral oil followed by alternating W640 and air bubbles, 12-17 healthy two-cell embryos from the previous day's injection are transferred into the recipient. The swollen ampulla is located and holding the oviduct between the ampulla and the bursa, a nick in the oviduct is made with a 28 g needle close to the bursa, making sure not to tear the ampulla or the bursa.

The pipette is transferred into the nick in the oviduct, and the embryos are blown in, allowing the first air bubble to escape the pipette. The fat pad is gently pushed into the peritoneum, and the reproductive organs allowed to slide in. The peritoneal wall is closed with one suture and the skin closed with a wound clip. The mice recuperate on a 37°C slide warmer for a minimum of four hours. The recipients are returned to cages in pairs, and allowed 19-21 days gestation. After birth, 19-21 days postpartum is allowed before weaning. The weanlings are sexed and placed into separate sex cages, and a 0.5 cm biopsy (used for genotyping) is snipped off the tail with clean scissors.

Genomic DNA is prepared from the tail snips using, for example, a QIAGEN DNEASY kit following the manufacturer's instructions. Genomic DNA is analyzed by PCR using primers designed to amplify a *Zwnt5* gene or a selectable marker gene that was introduced in the same plasmid. After animals are confirmed to be transgenic, they are back-crossed into an inbred strain by placing a transgenic female with a wild-type male, or a transgenic male with one or two wild-type female(s). As pups are born and weaned, the sexes are separated, and their tails snipped for genotyping. To check for expression of a transgene in a live animal, a partial hepatectomy is performed. A surgical prep is made of the upper abdomen directly below the zyphoid process. Using sterile technique, a small 1.5-2 cm incision is made below the sternum and the left lateral lobe of the liver exteriorized. Using 4-0 silk, a tie is made around the lower lobe securing it outside the body cavity. An atraumatic clamp is used to hold the tie while a second loop of absorbable Dexon (American Cyanamid; Wayne, N.J.) is placed proximal to the first tie. A distal cut is made from the Dexon tie and approximately 100 mg of the excised liver tissue is placed in a sterile petri dish. The excised liver section is transferred to a 14 ml polypropylene round bottom tube and snap frozen in liquid nitrogen and then stored on dry ice. The surgical site is closed with suture and wound clips, and the animal's cage placed on a 37°C heating pad for 24 hours post operatively. The animal is checked daily post operatively and the wound clips removed 7-10 days after surgery. The expression level of Zwnt5ε mRNA is examined for each transgenic mouse using an RNA solution hybridization assay or polymerase chain reaction.

In addition to producing transgenic mice that over-express Zwnt5, it is useful to engineer transgenic mice with either abnormally low or no expression of the gene. Such transgenic mice provide useful models for diseases associated with a lack of Zwnt5. As discussed above, Zwnt5 gene expression can be inhibited using anti-sense genes, ribozyme genes, or external guide sequence genes. To produce transgenic mice that under-express the *Zwnt5* gene, such inhibitory sequences are targeted to murine *Zwnt5* mRNA. Methods for producing transgenic mice that have abnormally low expression of a particular gene are known to those in the art [see, for example, Wu *et al.*, "Gene Underexpression in Cultured Cells and Animals by Antisense DNA and RNA Strategies," in *Methods in Gene Biotechnology*, pages 205-224 (CRC Press 1997)].

An alternative approach to producing transgenic mice that have little or no *Zwnt5* gene expression is to generate mice having at least one normal Zwnt5 allele replaced by a nonfunctional *Zwnt5* gene. One method of designing a nonfunctional *Zwnt5* gene is to insert another gene, such as a selectable marker gene, within a nucleic acid molecule that encodes murine Zwnt5. Standard methods for producing these so-called "knockout mice" are known to those skilled in the art [see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), and Wu *et al.*, "New Strategies for Gene Knockout," in *Methods in Gene Biotechnology*, pages 339-365 (CRC Press 1997)].

15. EDUCATIONAL KIT UTILITY OF ZWNT5 POLYPEPTIDES, POLYNUCLEOTIDES AND ANTIBODIES.

Polynucleotides and polypeptides of the present invention will additionally find use as educational tools as a laboratory practicum kits for courses related to genetics and molecular biology, protein chemistry and antibody production and analysis. Due to its unique polynucleotide and polypeptide sequence molecules of Zwnt5 can be used as standards or as "unknowns" for testing purposes. For example, Zwnt5 polynucleotides can be used as an aid, such as, for example, to teach a student how to prepare expression constructs for bacterial, viral, and/or mammalian expression, including fusion constructs, wherein Zwnt5 is the gene to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides; determining mRNA and DNA localization of Zwnt5 polynucleotides in tissues (i.e., by Northern and

Southern blotting as well as polymerase chain reaction); and for identifying related polynucleotides and polypeptides by nucleic acid hybridization.

Zwnt5 polypeptides can be used educationally as an aid to teach preparation of antibodies; identifying proteins by Western blotting; protein purification; determining the weight of expressed Zwnt5 polypeptides as a ratio to total protein expressed; identifying peptide cleavage sites; coupling amino and carboxyl terminal tags; amino acid sequence analysis, as well as, but not limited to monitoring biological activities of both the native and tagged protein (i.e., receptor binding, signal transduction, proliferation, and differentiation) *in vitro* and *in vivo*. Zwnt5 polypeptides can also be used to teach analytical skills such as mass spectrometry, circular dichroism to determine conformation, especially of the four alpha helices, x-ray crystallography to determine the three-dimensional structure in atomic detail, nuclear magnetic resonance spectroscopy to reveal the structure of proteins in solution. For example, a kit containing the Zwnt5 can be given to the student to analyze. Since the amino acid sequence would be known by the professor, the protein can be given to the student as a test to determine the skills or develop the skills of the student, the teacher would then know whether or not the student has correctly analyzed the polypeptide. Since every polypeptide is unique, the educational utility of Zwnt5 would be unique unto itself.

The antibodies that bind specifically to Zwnt5 can be used as a teaching aid to instruct students how to prepare affinity chromatography columns to purify Zwnt5, cloning and sequencing the polynucleotide that encodes an antibody and thus as a practicum for teaching a student how to design humanized antibodies. The *Zwnt5* gene, polypeptide or antibody would then be packaged by reagent companies and sold to universities so that the students gain skill in art of molecular biology. Because each gene and protein is unique, each gene and protein creates unique challenges and learning experiences for students in a lab practicum. Such educational kits, containing the *Zwnt5* gene, polypeptide or antibody, are considered within the scope of the present invention.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. An isolated polynucleotide, wherein said polynucleotide encodes a polypeptide comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2-20.

2. An isolated polypeptide, wherein said polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2-20.

3. An isolated antibody, wherein said antibody specifically binds to an amino acid sequence selected from the group consisting of SEQ ID NOs: 2-20.

SEQUENCE LISTING

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ttc cgg cat gag cgc tgg aac tgt agc ctg gag ggc agg atg ggc ctg      336
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ctc aag aga ggc ttc aaa gag aca gct ttc ctg tac gcg gtg tcc tct      384
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